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Published in:
Studies in Mycology

Link to article, DOI:
10.3114/sim.2011.69.05

Publication date:
2011

Document Version
Publisher's PDF, also known as Version of record

available online at www.studiesinmycology.org
doi:10.3114/sim.2011.69.05

Studies in Mycology 69: 57–80. 2011.

Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*

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Abstract: *Aspergillus* subgenus *Circumdati* section *Flavi* includes species with usually biseriate conidial heads, in shades of yellow-green to brown, and dark sclerotia. Several species assigned to this section are either important mycotoxin producers including aflatoxins, cyclopiazonic acid, ochratoxins and kojic acid, or are used in oriental food fermentation processes and as hosts for heterologous gene expression. A polyphasic approach was applied using morphological characters, extrolite data and partial calmodulin, β-tubulin and ITS sequences to examine the evolutionary relationships within this section. The data indicate that *Aspergillus* section *Flavi* involves 22 species, which can be grouped into seven clades. Two new species, *A. pseudocaelatus* sp. nov. and *A. pseudonomius* sp. nov. have been discovered, and can be distinguished from other species in this section based on sequence data and extrolite profiles. *Aspergillus pseudocaelatus* is represented by a single isolate collected from *Arachis burkartii* leaf in Argentina, is closely related to the non-aflatoxin producing *A. caelatus*, and produces aflatoxins B & G, cyclopiazonic acid and kojic acid, while *A. pseudonomius* was isolated from insects and soil in the USA. This species is related to *A. nomius*, and produces aflatoxin B_1 (but not G-type aflatoxins), chrysogine and kojic acid. In order to prove the aflatoxin producing abilities of the isolates, phylogenetic analysis of three genes taking part in aflatoxin biosynthesis, including the transcriptional regulator *aflR*, norsolonic acid reductase and O-methyltransferase were also carried out. A detailed overview of the species accepted in *Aspergillus* section *Flavi* is presented.

Key words: aflatoxin, Ascomycetes, *Aspergillus* section *Flavi,* β-tubulin, calmodulin, extrolites, ITS, polyphasic taxonomy.
Taxonomic novelties: *Aspergillus pseudocaelatus* Varga, Samson & Frisvad sp. nov., *Aspergillus pseudonomius* Varga, Samson & Frisvad sp. nov.

INTRODUCTION

Aspergillus section *Flavi* historically includes species with conidial heads in shades of yellow-green to brown and dark sclerotia. Isolates of the so-called domesticated species, such as *A. oryzae, A. sojae* and *A. tamarii* are used in oriental food fermentation processes and as hosts for heterologous gene expression (Campbell-Platt & Cook 1989). Genetically modified *A. oryzae* strains are used for the production of enzymes including lactase, pectin esterase, lipase, protease and xylanase (Pariza & Johnson 2001). Several species of section *Flavi* produce aflatoxins, among which aflatoxin B_1 is the most toxic of the many naturally occurring secondary metabolites produced by fungi. Aflatoxins are mainly produced by *A. flavus* and *A. parasiticus*, which coexist with and grow on almost any crop or food.

Several species have been described in the past which were assigned to *Aspergillus* section *Flavi* mainly based on traditional methods (morphological parameters, including colony diameter, colour and texture, size and texture of conidia and conidiophore structure; Klich 2002). However, species classification may be difficult due to extensive divergence of morphological characters produced by a high level of genetic variability (Kumeda & Asao 1996). Despite intense investigation, the taxonomy of this group of fungi is still highly complex. Recent data indicate that several of the species assigned to section *Flavi* cannot be distinguished based on morphological features alone (Frisvad *et al.* 2005, Pildain *et al.* 2008). Recently, a six-step molecular strategy using real-time PCR, RAPD and SmaI digestion of the nuclear DNA has been worked out to distinguish nine species of the section (Godet & Munaut 2010). In this study, we examined available isolates of the species proposed to belong to this section to clarify its taxonomic status. The methods used include sequence analysis of the ITS region (including intergenic spacer regions 1 and 2, and the 5.8 S rRNA gene of the rRNA gene cluster), and parts of the β-tubulin and calmodulin genes, macro- and micromorphological analysis, and analysis of extrolite profiles of the isolates. We also examined the presence of three aflatoxin biosynthetic genes in some aflatoxin-producing and non-producing isolates.

MATERIALS AND METHODS

Isolates

The strains used in this study are listed in Table 1. Sequence data of several other isolates available from GenBank database have also been used for constructing phylogenetic trees.

Morphological analysis

For macromorphological observations, Czapek Yeast Autolysate (CYA), Malt Extract Autolysate (MEA) agar, Yeast Extract Sucrose Agar (YES), Creatine Agar (CREA), and *Aspergillus flavus/parasiticus* Agar (AFPA) were used (Samson *et al.* 2004a). The isolates were inoculated at three points on each plate of each medium and incubated at 25 °C and 37 °C in the dark for 7 d. For micromorphological observations, microscopic mounts were made in lactic acid with cotton blue from MEA colonies and a drop of alcohol was added to remove air bubbles and excess conidia.

Table 1. *Aspergillus* isolates examined.

Name	Isolate	Source
A. albertensis	NRRL 20602^T = ATCC 58745	Human ear, Alberta, Canada
A. alliaceus	CBS 542.65^T = NRRL 4181	Soil, Australia
	CBS 536.65	Dead blister beetle *Macrobasis albida*, Washington, CO, USA
	CBS 612.78 = NRRL 5181	Buenos Aires, Argentina
A. arachidicola	CBS 117610^T = IBT 25020	*Arachis glabrata* leaf, CO, Argentina
	CBS 117615 = IBT 27178	*Arachis glabrata* leaf, CO, Argentina
A. avenaceus	CBS 109.46^T = IBT 4376	*Pisum sativum* seed, UK
	CBS 102.45	NCTC 6548
A. bombycis	CBS 117187 = NRRL 26010^T	Frass in a silkworm rearing house, Japan
A. caelatus	CBS 763.97^T = NRRL 25528	Soil, USA
	CBS 764.97 = NRRL 25404	Soil, USA
A. coremiiformis	CBS 553.77^T = NRRL 13756	Soil, Ivory Coast
A. fasciculatus	CBS 110.55^T	Air contaminant, Brazil
A. flavofurcatIs	CBS 484.65^T	Air contaminant, Brazil
A. flavus	CBS 100927^T	Cellophane, South Pacific Islands
	CBS 116.48	Unknown source, the Netherlands
	CBS 616.94	Man, orbital tumor, Germany
A. flavus var. *columnaris*	CBS 485.65^T	Butter, Japan
	CBS 117731	*Dipodomys spectabilis* cheek pouch, New Mexico, USA
A. kambarensis	CBS 542.69^T	Stratigraphic core sample, Japan
A. lanosus	CBS 650.74^T	Soil under *Tectona grandis*, Gorakhpur, India
A. leporis	CBS 151.66^T	Dung of *Lepus townsendii*, USA
	CBS 349.81	Soil, Wyoming, USA
A. minisclerotigenes	CBS 117633	*Arachis hypogaea* seed, FO, Argentina
	CBS 117635^T = IBT 27196	*Arachis hypogaea* seed, CD, Argentina
A. nomius	CBS 260.88^T = NRRL 13137	Wheat, USA
A. oryzae	CBS 100925^T	Unknown source, Japan
A. parasiticus	CBS 100926^T	*Pseudococcus calceolariae*, sugar cane mealy bug, Hawaii, USA
A. parasiticus var. *globosus*	CBS 260.67^T	Unknown source, Japan
A. parvisclerotigenus	CBS 121.62^T	*Arachis hypogaea*, Nigeria
A. pseudocaelatus	CBS 117616	*Arachis burkartii* leaf, CO, Argentina
A. pseudonomius	CBS 119388 = NRRL 3353	Diseased alkali bees, USA
A. pseudotamarii	CBS 766.97^T = NRRL 25517	Soil, USA
	CBS 765.97	Soil, USA
A. sojae	CBS 100928^T	Soy sauce, Japan
A. subolivaceus	CBS 501.65^T	Cotton, Lintafelt, UK
A. tamarii	CBS 104.13^T	Activated carbon
A. terricola	CBS 620.95	WB4858
	CBS 579.65^T	USA
A. terricola var. *americanus*	CBS 580.65^T	Soil, USA
	CBS 119.51	Japan
A. terricola var. *indicus*	CBS 167.63^T	Mouldy bread, Allahabad, India
A. thomii	CBS 120.51^T	Culture contaminant
A. togoensis	CBS 272.89^T	Seed, Central African Republic
A. toxicarius	CBS 822.72^T	*Arachis hypogaea*, Uganda
	CBS 561.82	Löss deposit, Nebraska, USA
A. zhaoqingensis	CBS 399.93^T	Soil, China

CBS = CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands. IBT = IBT Culture Collection of Fungi, Lyngby, Denmark. NRRL = USDA ARS Culture Collection, Peoria, USA. ATCC = American Type Culture Collection, Manassas, USA.

Extrolite analysis

The cultures were analysed according to the HPLC-diode array detection method of Frisvad & Thrane (1987, 1993) as modified by Smedsgaard (1997). The isolates were analysed on CYA and YES agar using three agar plugs (Smedsgaard 1997). Five plugs of each agar medium were taken and pooled together into same vial for extraction with 0.75 mL of a mixture of ethyl acetate/dichloromethane/methanol (3:2:1) (v/v/v) with 1 % (v/v) formic acid. The extracts were filtered and analysed by HPLC using alkylphenone retention indices and diode array UV-VIS detection as described by Frisvad & Thrane (1987), with minor modifications as described by Smedsgaard (1997).

Genotypic analysis

The cultures used for the molecular studies were grown on malt peptone (MP) broth using 1 % (w/v) of malt extract (Oxoid) and 0.1 % (w/v) bacto peptone (Difco), 2 mL of medium in 15 mL tubes. The cultures were incubated at 25 °C for 7 d. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol.) according to the instructions of the manufacturer. The ITS region and parts of the β-tubulin and calmodulin genes were amplified and sequenced as described previously (Varga *et al.* 2007a–c).

The presence of three genes taking part in aflatoxin biosynthesis has also been examined in some isolates. Part of the transcriptional regulator of aflatoxin biosynthesis, *aflR*, was amplified using the primers aflr-F (5'-GGGATAGCTGTACGAGTTGTGCCAG-3') and aflR-R (5'-TGGKGCCGACTCGAGGAAYGGGT-3') developed based on previously identified *aflR* sequences in the GenBank database. Part of the norsolonic acid reductase (*norA*, *aflE*; Yu *et al.* 2004) gene was amplified using the primers nor1 (5'-ACCGCTACGCCGGCACTCTCGGCA-3') and nor2 (5'-GTTGGCCGCCAGCTTCGACACAGC-3') developed by Geisen (1996). Part of the O-methyltransferase gene (*omtA*, *aflP*; Yu *et al.* 2004) was amplified using the primers omt1 (5'-GTGGACGGACCTAGTCCGACATCAC-3') and omt2 (5'-GTCGGCGCCACGCACTGGGTTGGGG-3') (Geisen 1996). Sequence analysis of the amplified products was carried out as described previously (Varga *et al.* 2007a).

DNA sequences were edited with the DNASTAR computer package. Alignments of the sequences were performed using MEGA v. 4 (Tamura *et al.* 2007). Phylogenetic analysis of sequence data was performed using PAUP v. 4.0b10 (Swofford 2000). Alignment gaps were treated as fifth character state, parsimony uninformative characters were excluded and all characters were unordered and equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option. To assess the robustness of the topology, 1000 bootstrap replicates were run by maximum parsimony (Hillis & Bull 1993). Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC, respectively) were also calculated. *Neopetromyces muricatus* CBS 112808^T was used as outgroup in the analyses of calmodulin, ITS and β-tubulin data sets, while *A. versicolor* SSRC 108 sequences were used as outgroups during analysis of *aflR* and *norA* sequences. No outgroup was used during the analysis of the *omtA* dataset, as sequences were not available from any other aflatoxin producing species outside *Aspergillus* section *Flavi*. Sequences were deposited at GenBank under accession numbers indicated on the figures.

RESULTS

Phylogenetic analysis

We examined the genetic relatedness of section *Flavi* isolates using sequence analysis of the ITS region of the ribosomal RNA gene cluster, and parts of the calmodulin and β-tubulin genes. During analysis of part of the β-tubulin gene, 561 characters were analysed, among which 223 were found to be phylogenetically informative. One of the 57 MP trees based on partial β-tubulin genes sequences is shown in Fig. 1 (tree length: 544 steps, consistency index: 0.7279, retention index: 0.9051). The calmodulin data set included 583 characters, with 221 parsimony informative characters. One of the 485 MP trees based on partial calmodulin gene sequences is shown in Fig. 2 (tree length: 557, consistency index: 0.7181, retention index: 0.9026). The ITS data set included 496 characters with 58 parsimony informative characters. One of the 235 MP trees is shown in Fig. 3 (tree length: 193, consistency index: 0.8446, retention index: 0.8592).

Phylogenetic analysis of ITS, calmodulin and β-tubulin sequence data indicated that the "*A. caelatus*" isolate CBS 117616 is closely related to, but phylogenetically distinct from *A. caelatus* (Figs 1–3). While all *A. caelatus* isolates known have came from soil, peanuts or tea fields located in Japan or USA, this isolate came from an *Arachis burkartii* leaf from Corrientes province, Argentina. This isolate also produces a set of different extrolites including aflatoxins B_1, B_2, G_1, G_2, kojic acid and cyclopazonic acid, while *A. caelatus* isolates produce kojic acid and aspirochlorin. Another isolate, "*A. nomius*" CBS 119388 (= NRRL 3353) was found to form a distinct clade on the trees based on calmodulin and β-tubulin sequence data (Fig. 1, 2). This isolate was also found to be different from *A. nomius* and *A. arachidicola* by physiological means; it produces chrysogine, kojic acid and aflatoxin B_1, similarly to *A. arachidicola*, which also produces aflatoxin G_1. In addition, *A. arachidicola* produces parasiticolide, ditryptophenaline and metabolite "NO2", the last one also being produced by isolate CBS 119388. *Aspergillus nomius* produces both B- and G-type aflatoxins, kojic acid, but not chrysogine. Based on phylogenetic analysis of calmodulin, β-tubulin, ITS and norsolonic acid reductase gene sequences, this new species includes several other isolates from insects and soil in Louisiana, Texas, Wyoming and Wisconsin in the USA (Peterson *et al.* 2001). Unfortunately, these isolates were not available for this study. The late C.W. Hesseltine (NRRL, Peoria USA) indicated in a personal communication to J.C. Frisvad, that he considered NRRL 3353 morphologically different from other *A. nomius*, which was backed up by differences in tolerance to low water activity. These observations should be further investigated.

The presence of 3 genes taking part in aflatoxin biosynthesis has also been examined in a set of isolates, including isolate CBS 117616 and several *A. caelatus* isolates. While isolate CBS 117616 carried homologs of all three examined genes, the *A. caelatus* isolates did not carry homologs of *aflR* and *norA* (Fig. 4). During analysis of the *aflR* dataset, 514 characters were analised, among which 113 were found to be phylogenetically informative. One of the 5 MP trees based on partial *aflR* genes sequences is shown in Fig. 5 (tree length: 464 steps, consistency index: 0.8836, retention index: 0.9339). The *norA* data set included 348 characters, with 40 parsimony informative characters. One of the 2 MP trees based on partial *norA* gene sequences is shown in Fig. 6 (tree length: 174, consistency index: 0.9138, retention index: 0.9032). The *omtA*

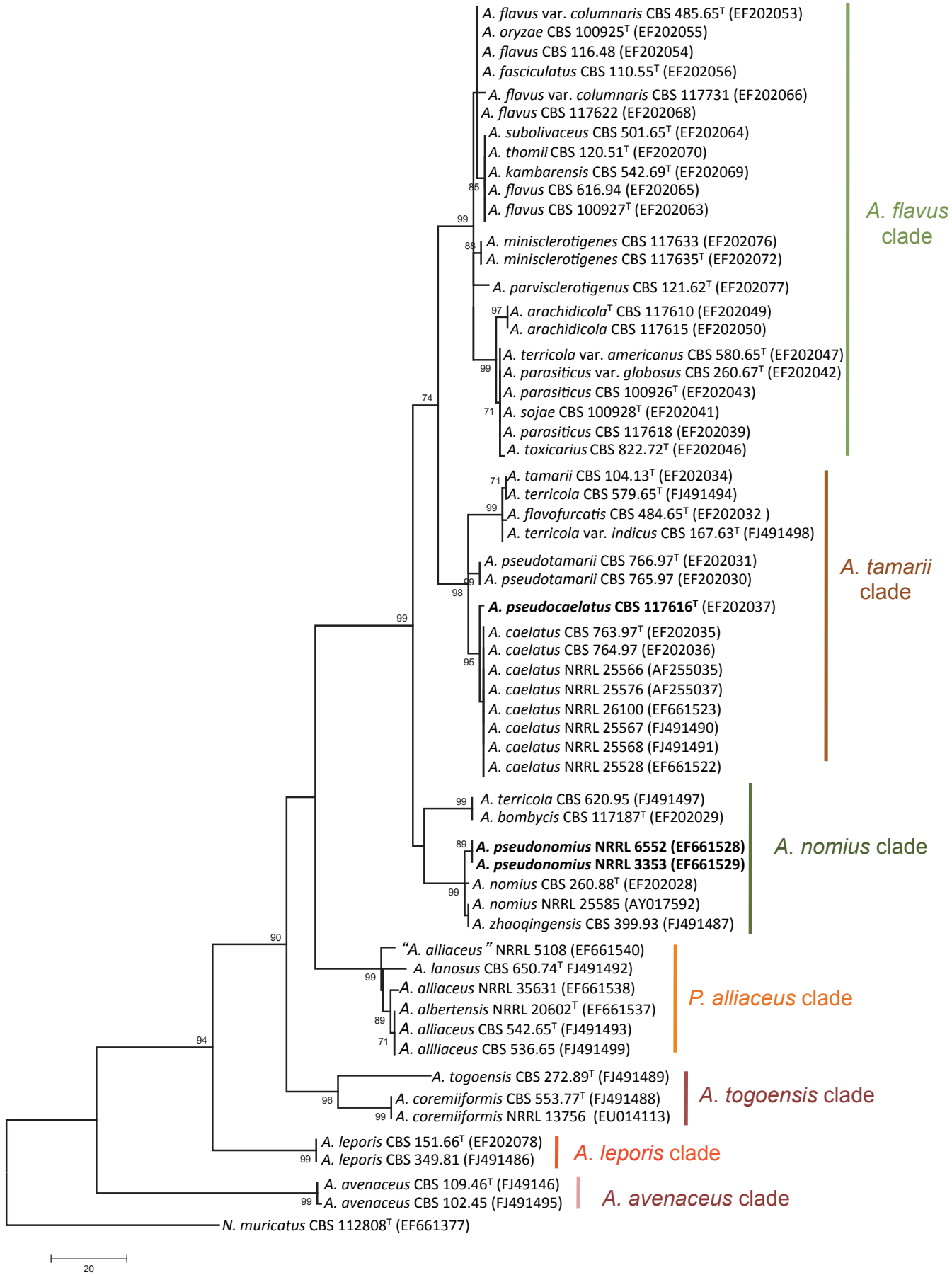

Fig. 1. Maximum parsimony tree based on β-tubulin sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated. *P.* = *Petromyces*. *N.* = *Neopetromyces*.

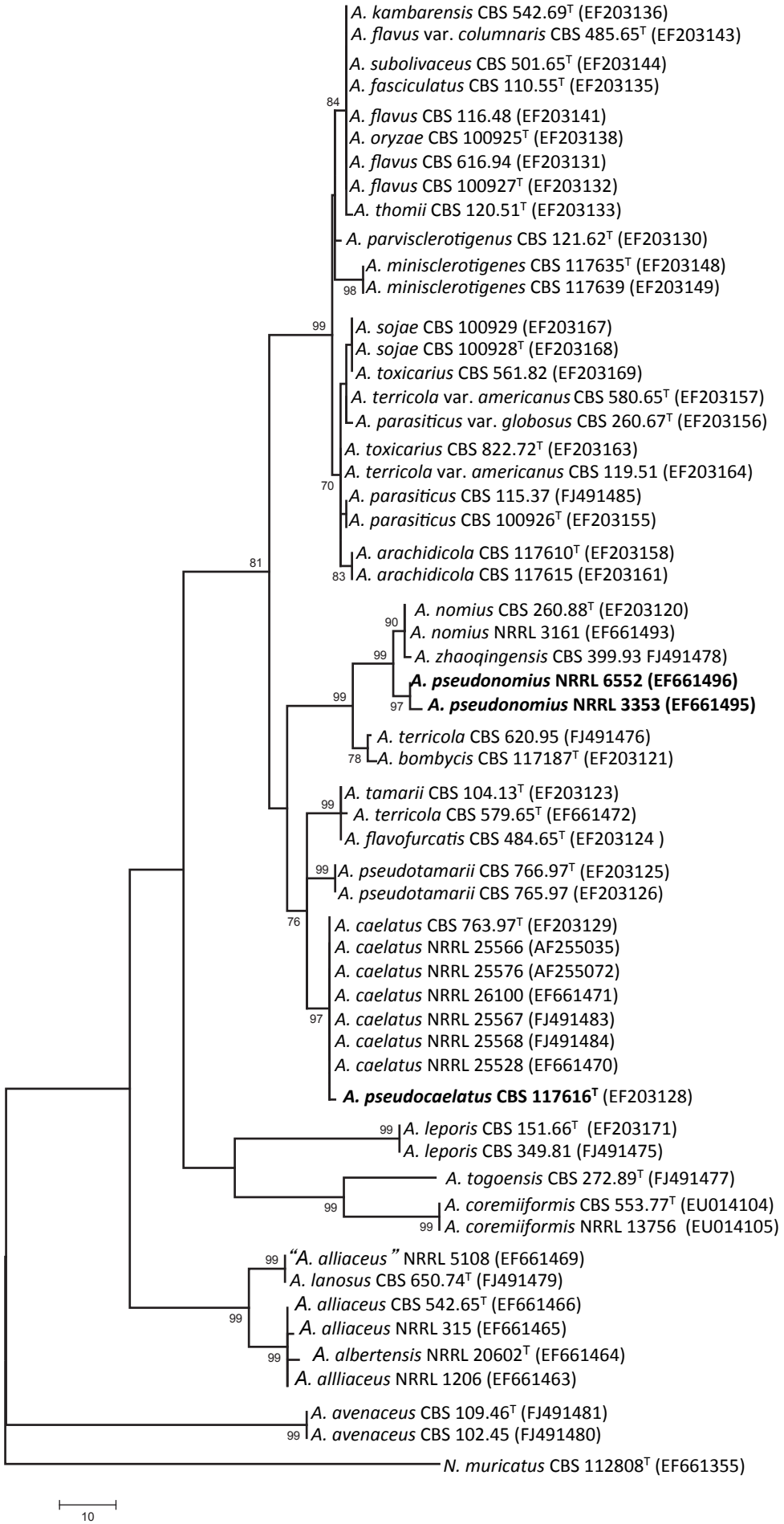

Fig. 2. Maximum parsimony tree based on calmodulin sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated. *N.* = *Neopetromyces*.

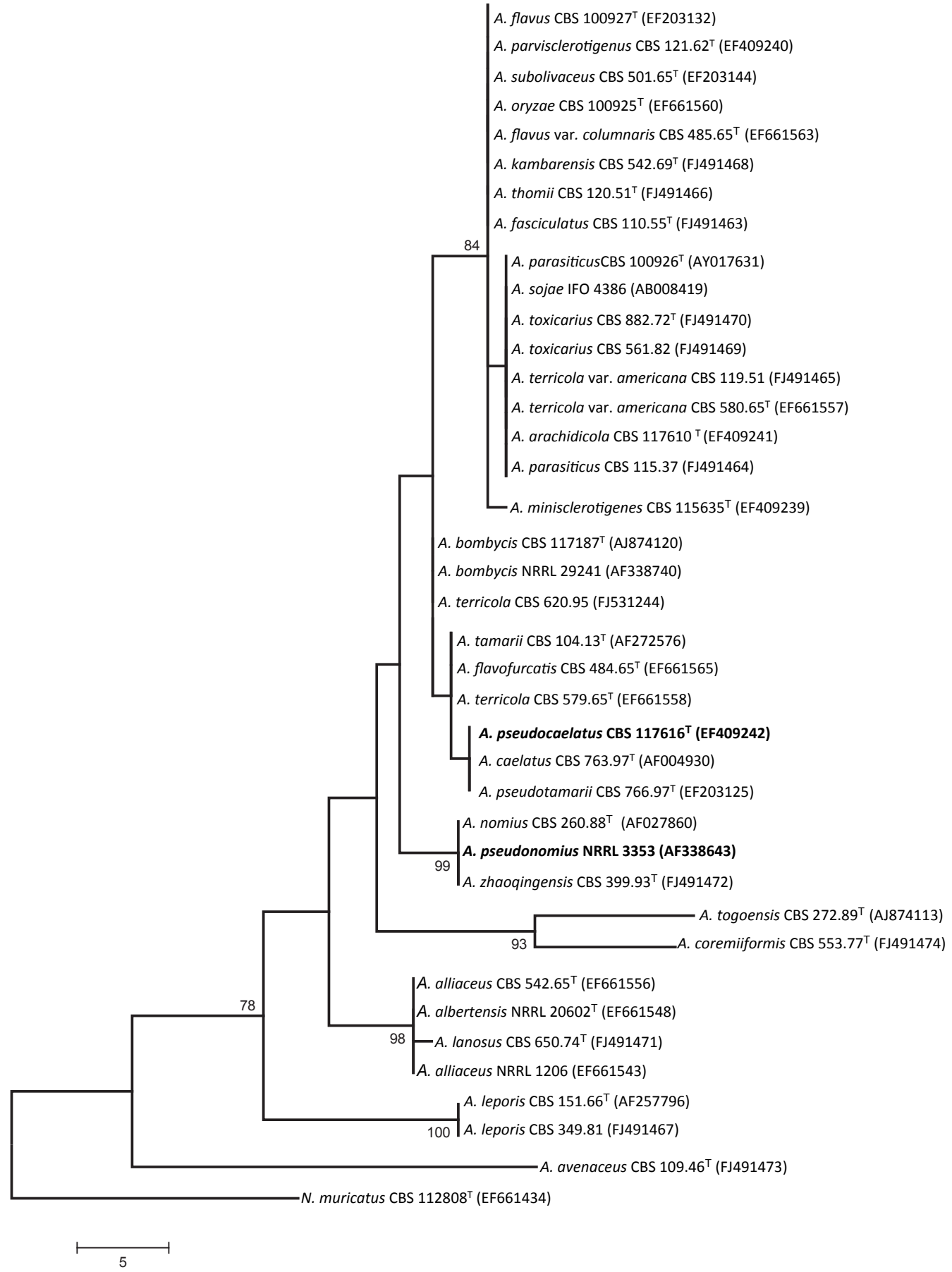

Fig. 3. Maximum parsimony tree based on ITS sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values. Only values above 70 % are indicated. *N.* = *Neopetromyces*.

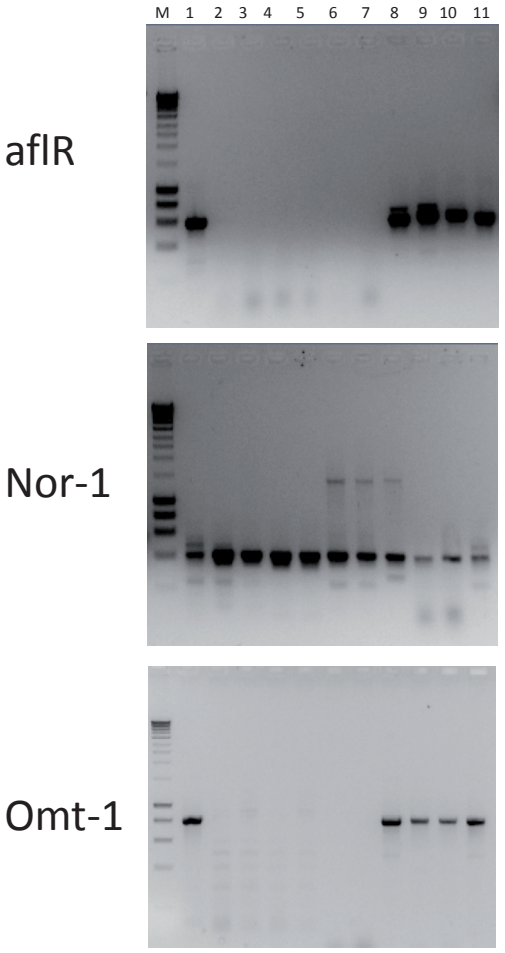

Fig. 4. PCR amplicons obtained using primer pairs developed for the *aflR*, *norA* and *omtA* genes in some isolates. M. 1 kb DNA ladder; 1. *A. pseudocaelatus* CBS 117616; 2–7. *A. caelatus* isolates (CBS 763.97, CBS 764.97, NRRL 25566, NRRL 25567, NRRL 25568 and NRRL 25569); 8. *A. minisclerotigenes* CBS 117633; 9. *A. arachidicola* CBS 117610; 10. *A. parvisclerotigenus* CBS 121.62; 11. *A. bombycis* NRRL 29236.

data set included 731 characters, with 136 parsimony informative characters. One of the 12 MP trees based on partial *omtA* gene sequences is shown in Fig. 7 (tree length: 386, consistency index: 0.7876, retention index: 0.8019). Isolate CBS 117616 was related to *A. pseudotamarii* based on *aflR* and *omtA* sequence data (Figs 5, 7), while the *norA* data set revealed that it is more closely related to *A. caelatus* (Fig. 6). Isolate CBS 119388 was related to, but distinct from *A. nomius* based on all trees. We propose the names *Aspergillus pseudocaelatus* and *A. pseudonomius* for these two new species.

Aspergillus pseudocaelatus Varga, Samson & Frisvad, **sp. nov.** MycoBank MB560397. Fig. 8.

Aspergillo caelato morphologice valde similis, sed aflatoxina (B & G), acor cyclopiazonicus et acor kojicus formantur.

Colonies on YES, MEA, OA and CYA attain a diam of 6–6.5 cm in 7 d at 25 °C; growing rapidly on CYA at 37 °C, with a diam of 6–7 cm. On CREA a typical acid production. Colony surface velvety with abundant conidial heads, olive to olive brown en masse. Reverse greenish yellow without diffusible pigments. Sclerotia not observed. Conidial heads uniseriate or biseriate. Stipes hyaline, smooth-walled, 5–8 µm wide variable in length, mostly (250–)400–600(21000) µm;

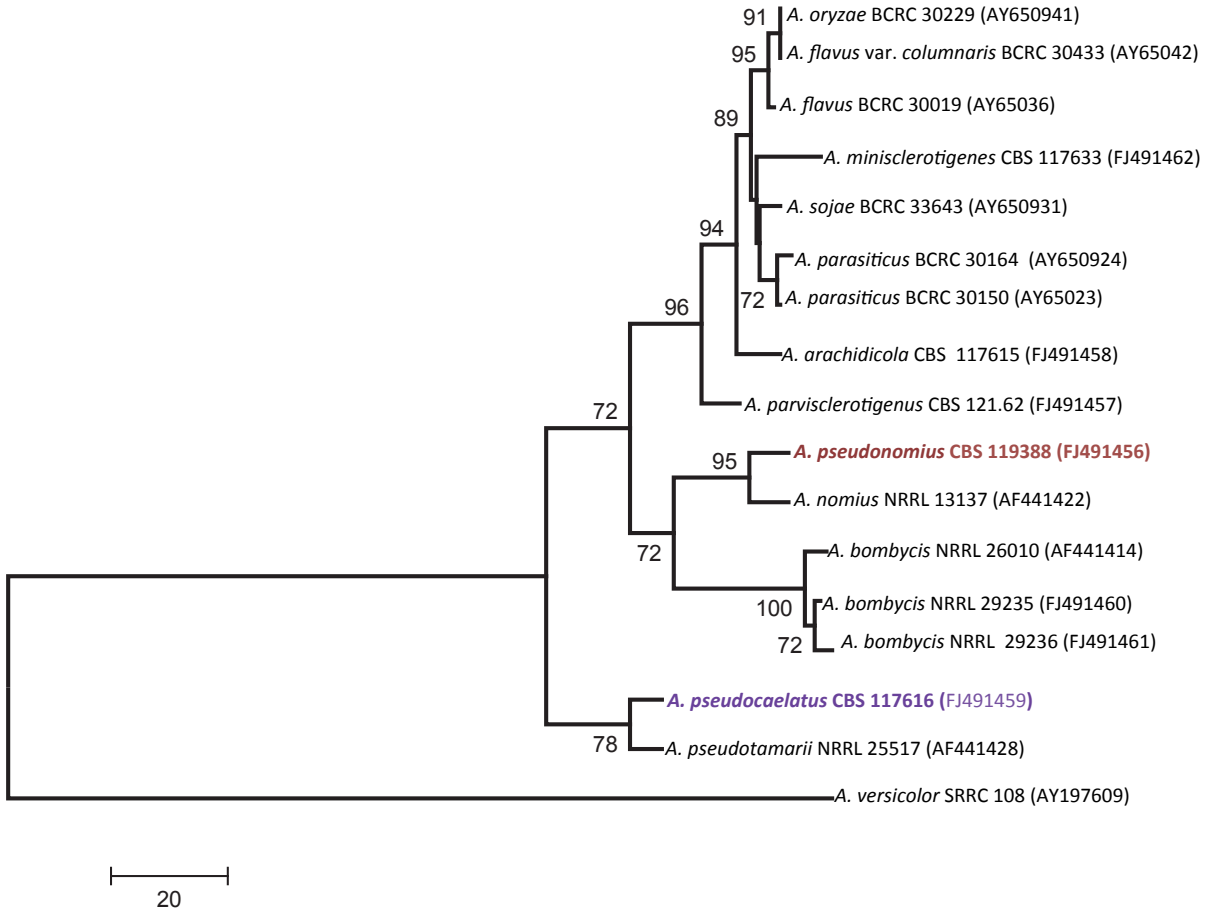

Fig. 5. Maximum parsimony tree based on *aflR* sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated.

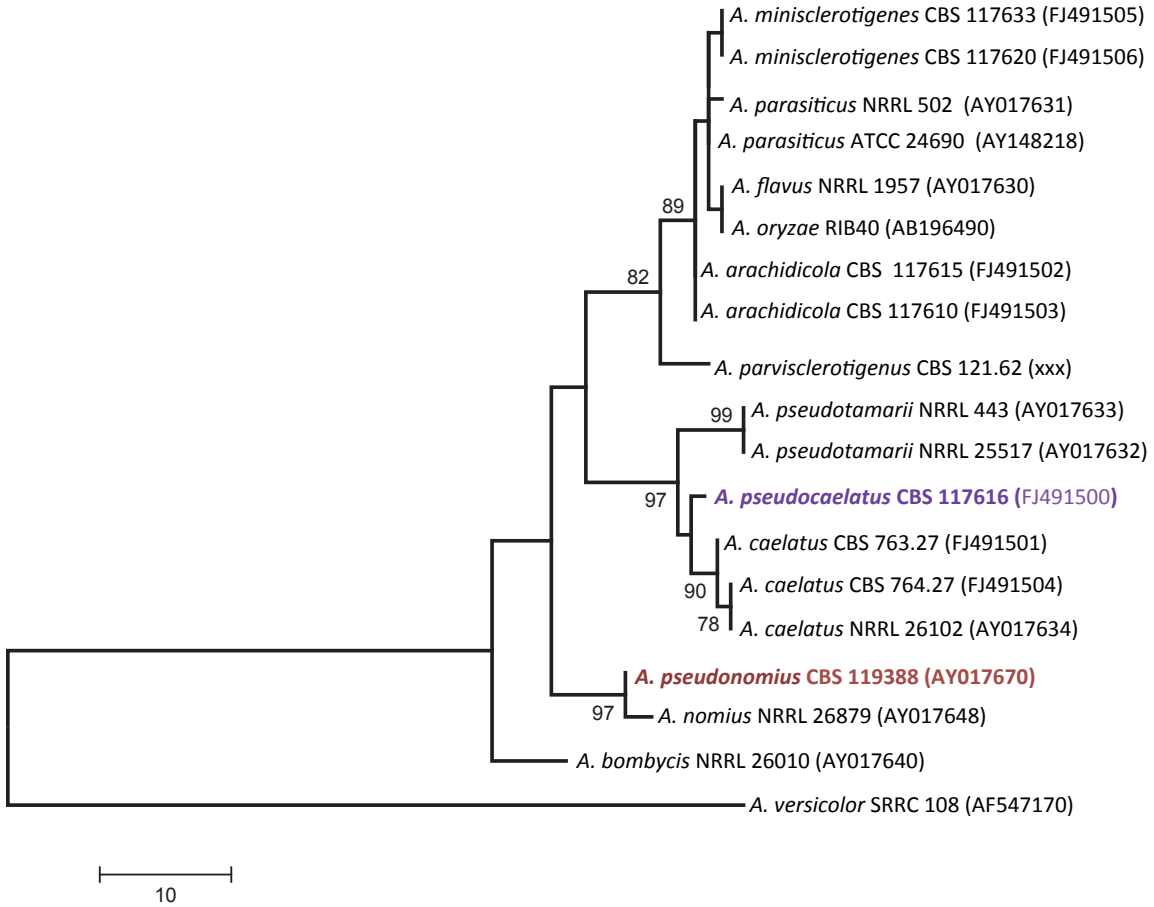

Fig. 6. Maximum parsimony tree based on *norA* sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated.

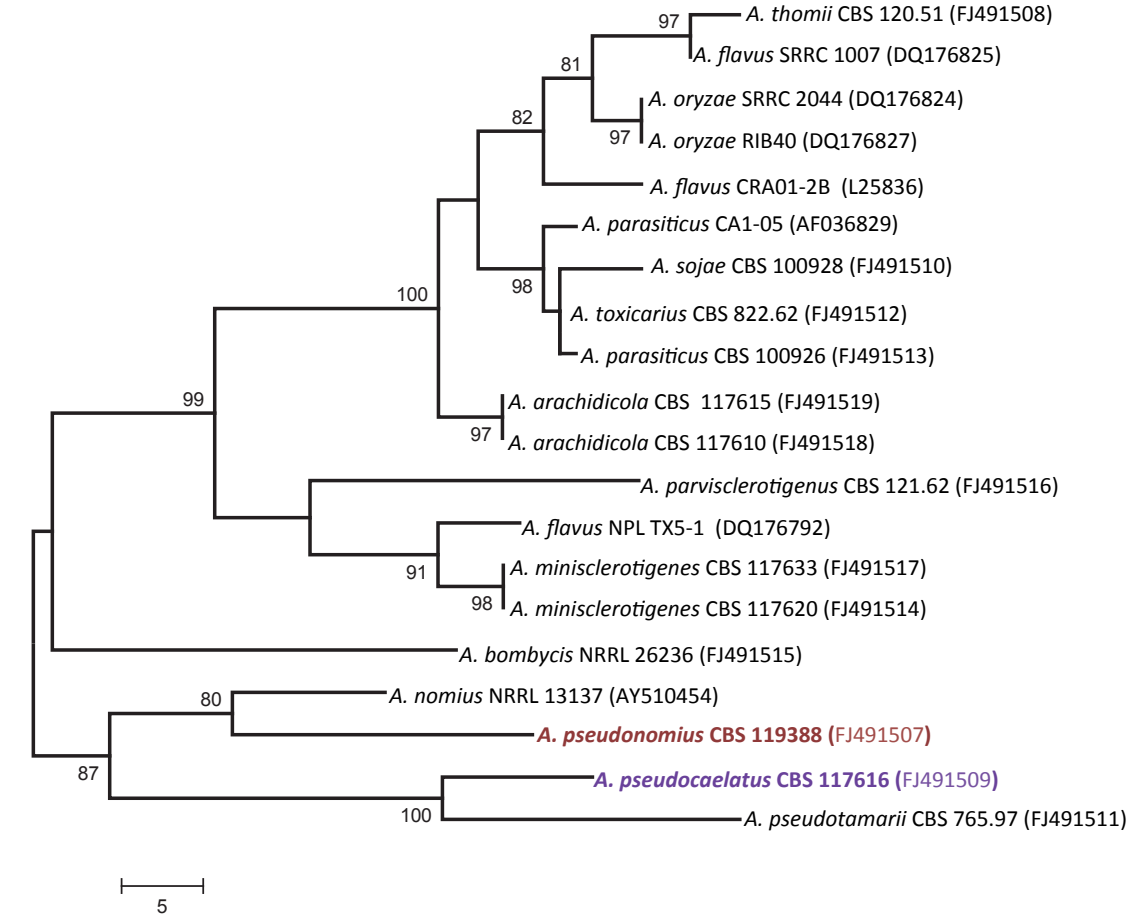

Fig. 7. Maximum parsimony tree based on *omtA* sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated.

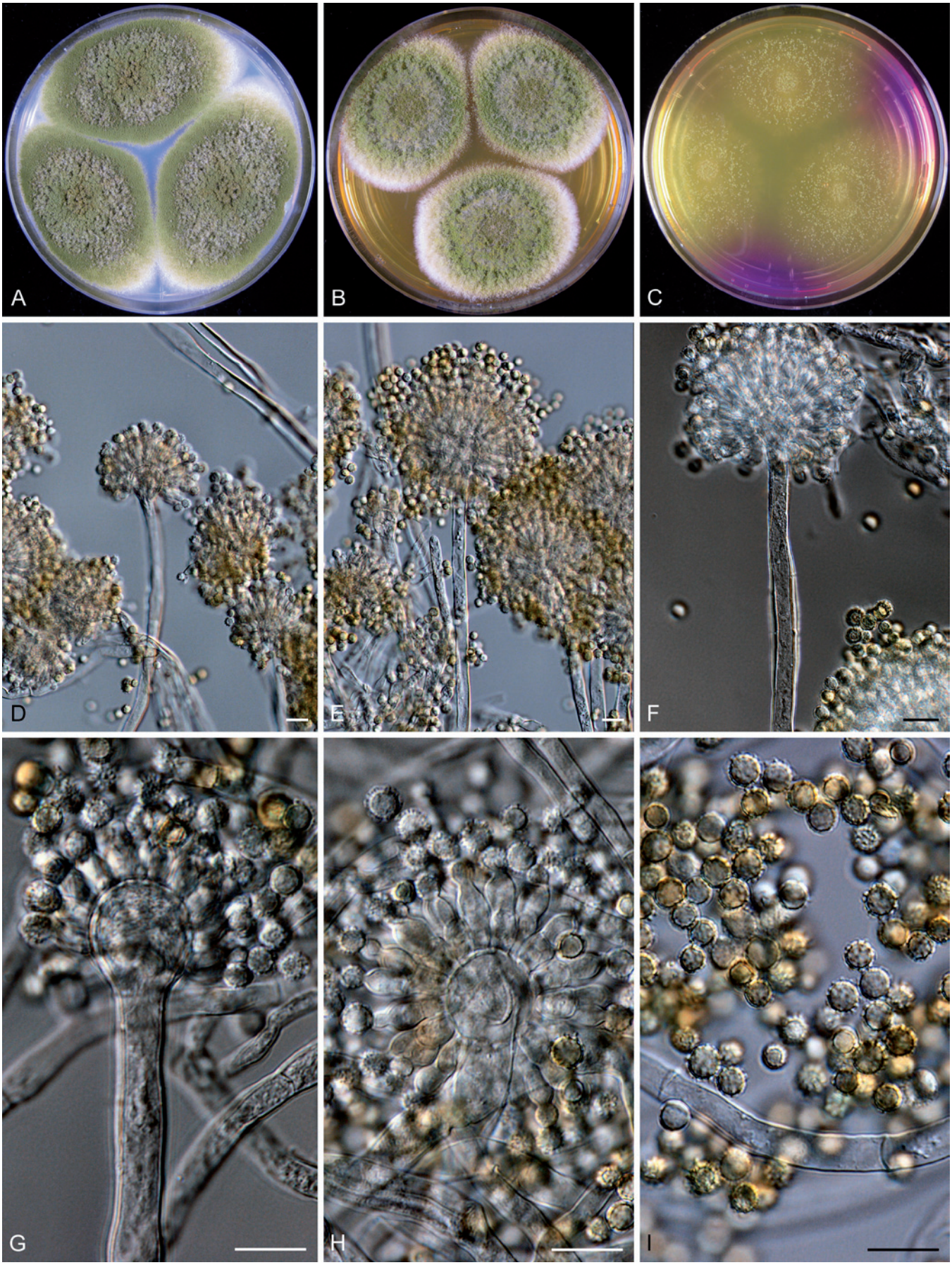

Fig. 8. *Aspergillus pseudocaelatus* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

Vesicles globose to subglobose, 17–22 mm in diam. Conidia globose to subglobose, echinulate, greenish, 4.5–5 µm. Isolates grow well at 25, 37 and 42 °C.

Extrolites: strains of *A. pseudocaelatus* produce aflatoxins B_1,B_2 & G_1,G_2, cyclopiazonic acid and kojic acid.

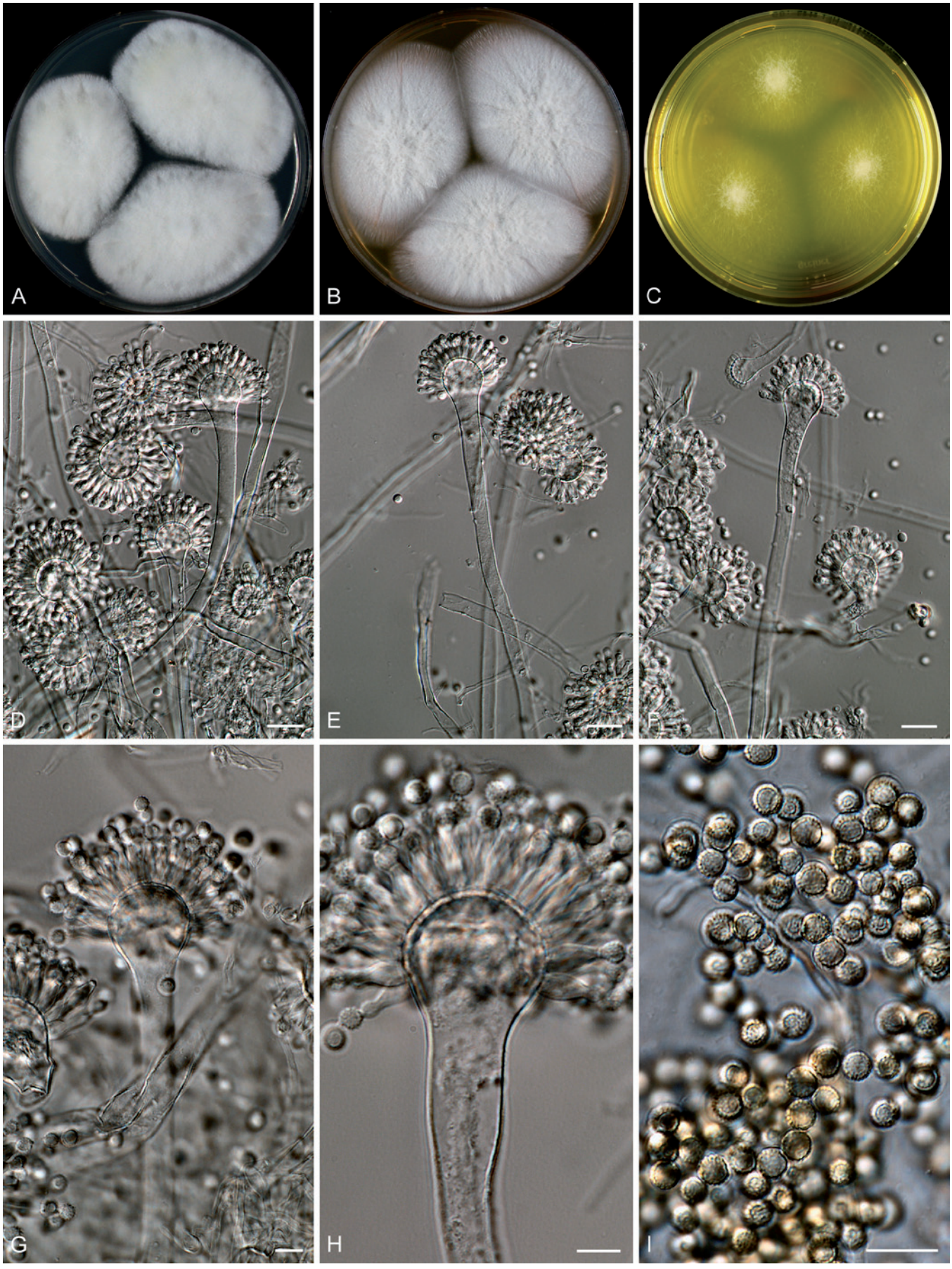

Fig. 9. *Aspergillus pseudonomius* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

Typus: **Argentina**, Corrientes province; isolated from an *Arachis burkartii* leaf, Isolated by B. Pildain (CBS H-20632 -- holotypus, culture ex-type CBS 117616).

Aspergillus pseudocaelatus is represented by a single isolate collected from an *Arachis burkartii* leaf in Argentina. It is closely related to the non-aflatoxin producing *A. caelatus,* and produces aflatoxins B & G, cyclopiazonic acid and kojic acid. *Aspergillus caelatus* isolates produce kojic acid and aspirochlorin

Aspergillus pseudonomius Varga, Samson & Frisvad, **sp. nov.** MycoBank MB560398. Fig. 9.

Aspergillo nomio morphologice valde similis, sed aflatoxinum B1 (neque aflatoxina typi G), chrysoginum et acor kojicus formantur.

Colonies on YES, MEA, OA and CYA attain a diam of 6–6.5 cm in 7 d at 25 °C; growing rapidly on CYA at 37 °C, with a diam of 6–7 cm. On CREA a typical acid production. Colony surface floccose with dominant aerial mycelium with poor sporulation. Reverse not coloured. Sclerotia not observed. Conidial heads uniseriate. Stipes hyaline, smooth, variable in length, mostly (250–)400–600(21000) µm; diam just below vesicles 5–8 mm. Vesicles globose to subglobose, 15–30 µm in diam, fertile upper 75 % of their surface; Conidia globose to subglobose, echinulate, greenish, 4–5 µm. Isolates grow well at 25, 37 and 42 °C.

Extrolites: strains of *A. pseudonomius* produce aflatoxin B_1, chrysogine and kojic acid.

Typus: **USA**, was isolated diseased alkali bees (CBS H-20633 -- holotypus, culture ex-type CBS 119388^T = NRRL 3353).

Aspergillus pseudonomius was isolated from insects and soil in the USA. It is related to *A. nomius,* and produces aflatoxin B_1 (but not G-type aflatoxins), chrysogine and kojic acid.

An overview of *Aspergillus* section *Flavi*

In this study, we used sequence data from three loci to clarify the taxonomy of this section. Based on our phylogenetic analysis of calmodulin and ITS sequence data, *Aspergillus* section *Flavi* includes 7 main clades (Figs 1–3) with 20 or more taxa. The main clades isolates form well-defined subclades on the trees based on both β-tubulin and calmodulin sequence data. However, they are represented mostly by a single isolate *e.g. A. coremiiformis, A. togoensis.* Further collections and studies are needed to clarify if they represent separate species.

Figures 10–12 show the colonies of the accepted species on CYA, MEA and YES which are growing all well on these media, mostly reaching a diam of 6 cm within 7 d. However the colony colour differences are distinct allowing to recognise the less common species from the typical yellow-green colonies of *A. flavus* (Fig. 10 A)*, A. arachdicicola* (Fig. 10D)*, A. caelatus* (Fig. 10E)*, A. pseudocaelatus* (Fig. 11C) and *A. parasiticus* (Fig. 11H). Other species are brown (*A. tamarii* Fig. 12E) or have a less pronounced colony colours due to poor sporulation or the presence of dark sclerotia. Conidial shape and ornamentation of the species are depicted in Figs 13, 14. Conidia of species in section *Flavi* are mostly globose and rough to echinulate. The conidial shape of most species is globose with rough to distinct ornamentation. The conidial shape of *A. togoensis* and *A. coremiiformis* is irregularly shaped, smooth-walled and larger than those produced by other taxa in section *Flavi*. The conidia of *A. leporis*, and *Petromyces alliaceus* and *P. albertensis* are globose but relatively small.

Aspergillus avenaceus is the most basal member of the section. Isolates of this species produce very long black sclerotia and long conidiophores (Kozakiewicz 1989), and have Q-10 as their main ubiquinones (Kuraishi *et al.* 1990). Samson (1979) and Kozakiewicz (1989) suggested that *A. avenaceus* might be related to *A. alliaceus* based on morphological features; however, sequence data do not support this view. *Aspergillus avenaceus* has been found to produce avenaciolide, a water-insoluble bis-g-lactone antibiotic which possesses antifungal activity, and is a specific inhibitor of glutamate transport in rat liver mitochondria (Brookes *et al.* 1963, McGivan & Chapell 1970).

Another clade includes *A. leporis* isolates. This species is characterised by a Q-10 ubiquinone system, conidial heads in shades of olive, and white-tipped cinnamon coloured sclerotia (Christensen 1981, Kuraishi *et al.* 1990). Interestingly, isolates of this species produce sclerotia on rabbit dung, but not on CYA or MEA plates (Wicklow 1985). The sclerotia of *A. leporis* contain the antiinsectan N-alkoxypyridone metabolite, leporin A (Tepaske *et al.* 1991), which has been found to be effective in controlling Lepidopteran insect pests (Dowd *et al.* 1994).

Aspergillus coremiiformis and *A. togoensis* are related based on all sequence data. The species are characterised by the formation of synnemata as illustrated by the ex-type strain of *A. togoensis* (CBS 272.89) (Fig. 15). The close relationship of *A. coremiiformis* to species of section *Flavi* was also suggested by Samson (1979), Christensen (1981), and Roquebert & Nicot (1985) based on morphological features. The latter authors stated that "*Stilbothamnium nudipes* (= *A. coremiiformis*) differs from *A. tamarii* only by having septate phialides" (Roquebert & Nicot 1984). Molecular data also indicated previously that these species have affinities to section *Flavi* (Dupont *et al.* 1990, Rigó *et al.* 2002, Frisvad *et al.* 2005). The observation that an *A. togoensis* isolate produces sterigmatocystin, an intermediate of the aflatoxin biosynthetic pathway also indicates that this species is a member of *Aspergillus* section *Flavi* (Wicklow *et al.* 1989). Recently, *A. togoensis* was also found to be able to produce aflatoxin B_1 and O-methyl-sterigmatocystin (Rank *et al.* 2011). There are only a few isolates of *A. togoensis* and *A. coremiiformis* known and more strains should be made available to elucidate the relationship between these two taxa.

Aspergillus alliaceus together with *A. lanosus* and *A. albertensis* form another clade on all trees. Thom & Raper (1945) and Kozakiewicz (1989) assigned the *A. alliaceus* species to the *A. wentii* species group (*Aspergillus* section *Wentii*) based mainly on morphological features, while later the teleomorphic *Petromyces* genus was assigned to *Aspergillus* section *Circumdati* (Gams *et al.* 1985, Samson 1994). Varga *et al.* (2000a, b) and Frisvad & Samson (2000) found that *A. lanosus*, and anamorphs of *Petromyces alliaceus* and *P. albertensis* are closely related to *Aspergillus* section *Flavi*. *Aspergillus alliaceus* is of world-wide distribution. This species was first identified as a wound parasite of onion bulbs (Raper & Fennell 1965), and is mainly isolated from grassland soils, nuts, and from air (Christensen & Tuthill 1985, Kozakiewicz 1989). *Aspergillus albertensis* was isolated from a man's ear swab in Canada (Tewari 1985). While *A. alliaceus* produces determinate ellipsoidal black stromata, *A. albertensis* produces indeterminate irregularly shaped grey stromata (Tewari 1985). Both *A. alliaceus* and *A. albertensis* are homothallic, and produce ascospores in ascocarps embedded in stromata after relatively long incubation period (after about 8 wk in *A.*

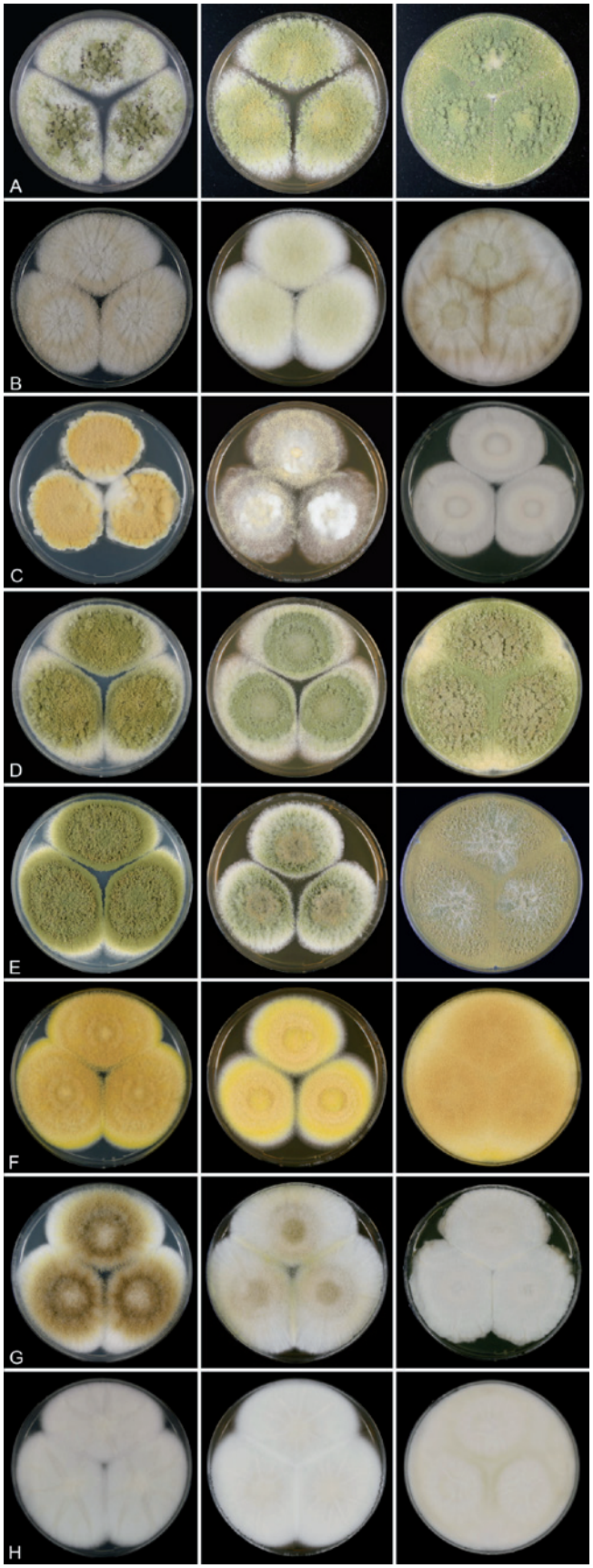

Fig. 10 Colonies of the various species of section *Flavi* on CYA. MEA and YES (7 d at 25 °C), A. *Aspergillus flavus* 100927, B. *A. avenaceus* 109.46, C. *A. coremiiformis* 553.77, D. *A. arachidicola* 117610, E. *A. caelatus* 763.27, F. *A. lanosus* 650.74, G. *A. bombycis* 117187, H. *A. leporis* 151.66.

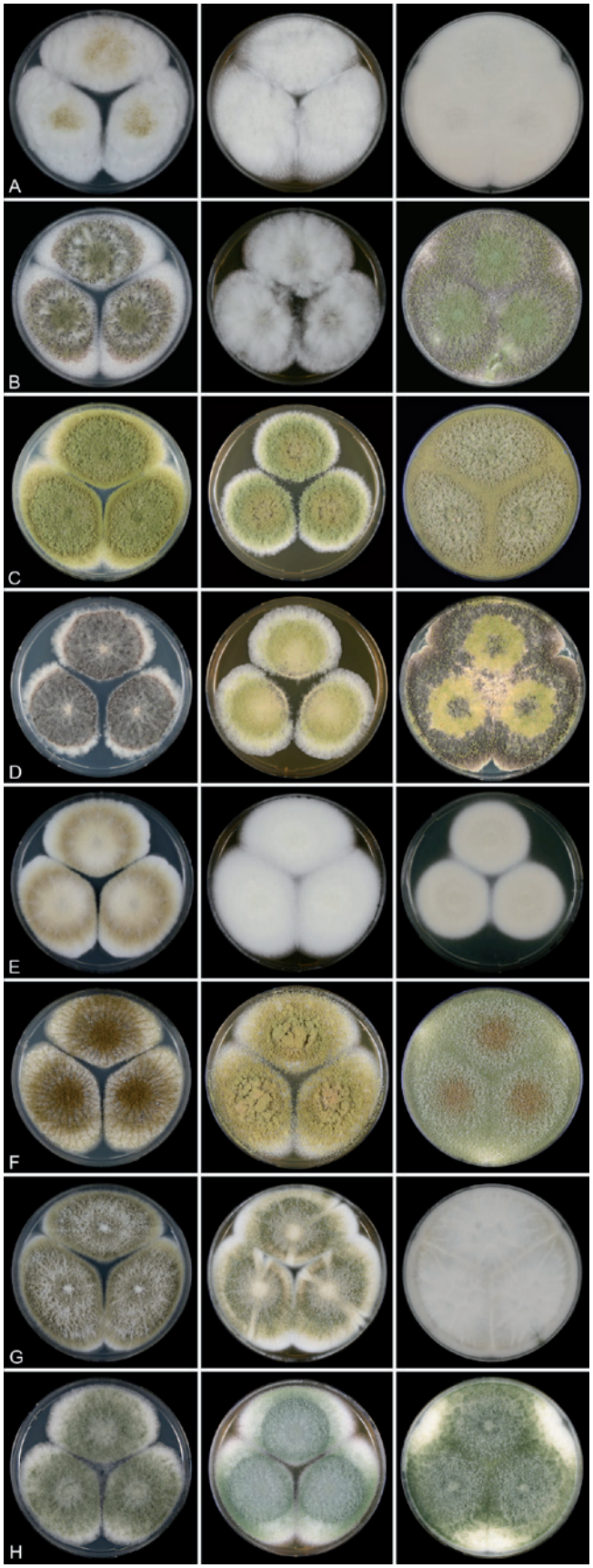

Fig. 11. Colonies of the various species of section *Flavi* on CYA. MEA and YES (7 d at 25 °C). A. *Aspergillus nomius* 119388, B. *A. minisclerotium* 117635, C. *A. pseudocaelatus* 117616, D. *A. parvisclerotigenus* 121.62, E. *A. oryzae* 100925, F. *A. pseudotamarii* 766.97, G. *A. sojae* 100928, H. *A. parasiticus* 100926.

albertensis, and after 3–4 mo in *A. alliaceus*; Fennell & Warcup 1959, Tewari 1985). Ascospores were found to be smooth with a fine ridge (Tewari 1985). Sequence analyses of multiple loci indicate that *A. albertensis* is a synonym of *A. alliaceus* (Figs 1–3; Varga *et al.* 2000, Peterson 2000, McAlpin & Wicklow 2005, Peterson 2008). Several isolates of these species are able to produce ochratoxin A & B, and are considered to be responsible for ochratoxin contamination of figs (Varga *et al.* 1996, Bayman *et al.* 2002). *Aspergillus alliaceus* isolates

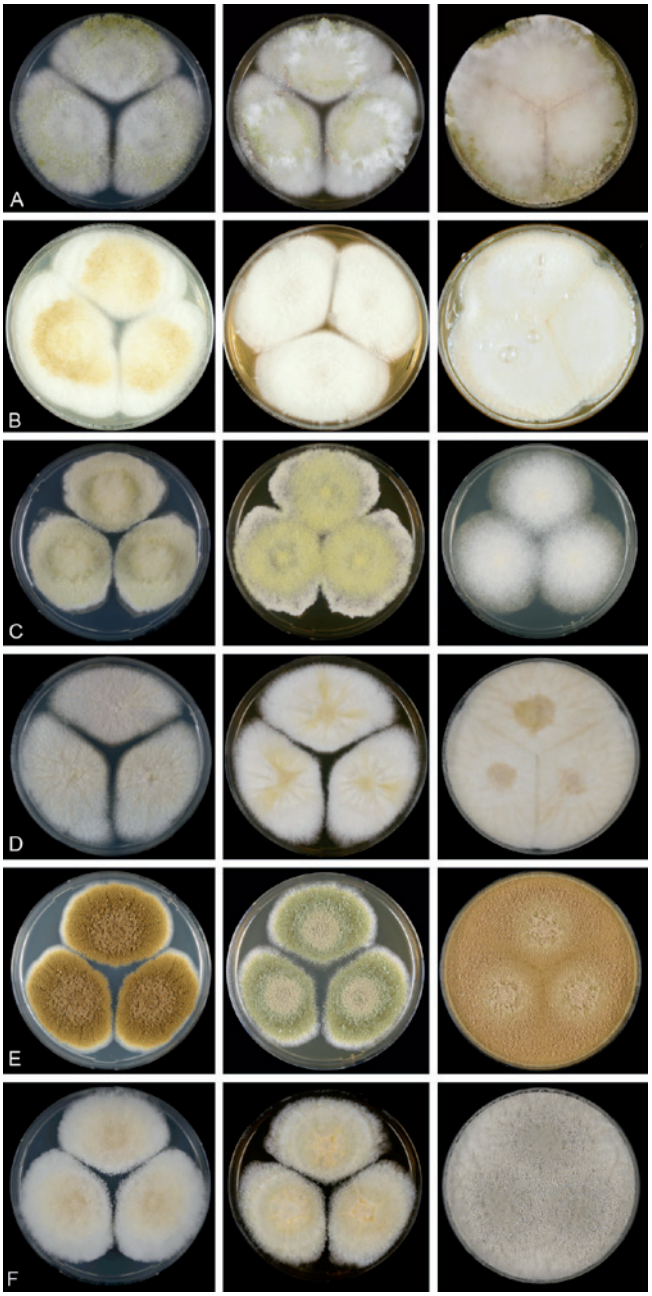

Fig. 12. Colonies of the various species of section *Flavi* on CYA. MEA and YES (7 d at 25 °C). A. *Aspergillus nomius* 260.88, B. *A. pseudonomius* C. *A. togoensis* 272.89, D. *Petromyces alliaceus* 110.26, E. *A. tamarii* 104.13, F. *P. albertensis* ATCC 58745.

are also able to produce ochratoxins under "*ex vivo*" conditions (Klich *et al.* 2009). Consequently, ochratoxins were suggested to act as potential virulence factors during pathogenesis. *Aspergillus alliaceus* has also been encountered in human infections including otorrhea (Koenig *et al.* 1985), invasive aspergillosis (Balajee *et al.* 2007) and pulmonary infection (Ozhak-Baysan *et al.* 2010). *Aspergillus alliaceus* was shown to exhibit reduced *in vitro* susceptibilities to amphotericin B and caspofungin (Balajee *et al.* 2007). Stromata of *A. alliaceus* strains contain compounds exhibiting insecticidal properties (Laakso *et al.* 1994, Nozawa *et al.* 1994), and asperlicins, potent cyclic peptide antagonists of cholecystokinin (Liesch *et al.* 1988). *Aspergillus alliaceus* strains are also used for steroid and alkaloid transformations (Burkhead *et al.* 1994, Sanchez-Gonzalez & Rosazza 2004), and for the production of pectin degrading enzyme preparations (Mikhailova *et al.* 1995).

Another clade includes *A. nomius, A. pseudonomius* and *A. bombycis* isolates. *Aspergillus nomius* and *A. bombycis* produce both aflatoxins B and G, *A. pseudonomius* produces only aflatoxin B_1, while none of them produce cyclopiazonic acid (Peterson *et al.* 2001, Table 2). *Aspergillus bombycis* was isolated from silkworm-rearing houses in Japan and Indonesia, while *A. nomius* is more widespread: it was originally isolated from mouldy wheat in the USA, and later from various substrates in India, Japan and Thailand. *Aspergillus nomius* is often associated with insects such as alkali bees (Hesseltine *et al.* 1970, Kurtzman *et al.* 1987) and termites (Rojas *et al.* 2001) and is frequently isolated from insect frass in silkworm-rearing houses in eastern Asia (Ito *et al.* 1998, Peterson *et al.* 2001). In addition soil populations in agricultural fields (Horn & Dorner 1998, Ehrlich *et al.* 2007) suggest that *A. nomius* might contribute to aflatoxin contamination of crops. *Aspergillus nomius* has been reported from tree nuts (Olsen *et al.* 2008, Doster *et al.* 2009), sugarcane (Kumeda *et al.* 2003) and an assortment of seeds and grain (Kurtzman *et al.* 1987, Pitt *et al.* 1993, Kumeda *et al.* 2003).

A recent study of soil samples from Thailand demonstrated that *A. nomius* is more widespread than may be commonly thought; it can be the predominant aflatoxin-producing *Aspergillus* species at certain geographic locations and must be considered a potential etiological agent of aflatoxin contamination events due to its ability to produce large quantities of aflatoxins (Ehrlich *et al.* 2007). For example, *A. nomius* accounted for > 9 % of section *Flavi* isolates from cornfield soils Iran (Razzaghi-Abyaneh *et al.* 2006). Recently, Olsen *et al.* (2008) have observed that *A. nomius* is an important producer of aflatoxins in Brazil nuts. *Aspergillus nomius* was recently identified from keratitis cases in India (Manikandan *et al.* 2009). Peterson *et al.* (2001) observed cryptic recombination in *A. nomius* populations using multilocus sequence data. Recently, Horn *et al.* (2010) identified the sexual state of *A. nomius* and named it as *Petromyces nomius*. An incubation period of 5 to 10 mo was needed for the formation of ascocarps within stromata. Ascocarp and ascospore morphology in *A. nomius* were similar to that of A. *flavus* and *A. parasiticus* and differences between teleomorphs were insufficient for species separation. The majority of *A. nomius* strains were either MAT1-1 or MAT1-2, but several strains contained both genes. MAT1-1/MAT1-2 strains were self sterile and capable of mating with both MAT1-1 and MAT1-2 strains; hence, *A. nomius* appears to be functionally heterothallic (Horn *et al*. 2010).

Aspergillus pseudonomius has so far only been isolated from insects and soil in the USA. *Aspergillus terricola* isolate CBS 620.95 (=WB4858), which was Blochwitz's strain of *A. luteo-virescens* (Raper & Fennell 1965), belongs to the *A. bombycis* species. *Aspergillus zhaoqingensis* was isolated from soil in China (Sun & Qi 1991), and found to be able to produce kojic acid, aspergillic acid, aflatoxin B_2 and tenuazonic acid, like most strains of *A. nomius* (unpubl. data). Molecular data indicate that *A. zhaoqingensis* is a synonym of *A. nomius* (Figs 1–3). Recent data indicate that *A. nomius* is a paraphyletic group likely to contain several other species (Egel *et al.* 1994, Cotty & Cardwell 1999, Kumeda *et al.* 2003, Ehrlich *et al.* 2003, Peterson 2008, Doster *et al.* 2009). Based on sequence alignments for three DNA regions the *A. nomius* isolates could be separated into three well-supported clades (Ehrlich *et al.* 2007). Further studies on these clades are in progress.

The "*A. tamarii*" clade contains species with ubiquinone system Q-10(H_2), and conidia in shades of olive to brown (Kuraishi *et al.* 1990, Rigó *et al.* 2002). This clade includes *A. tamarii* and its synonyms *A. terricola, A. terricola* var. *indicus* and *A. flavofurcatis, A. caelatus*, and two aflatoxin producing species: *A. pseudotamarii* and *A. pseudocaelatus*. *Aspergillus tamarii* isolates are widely used

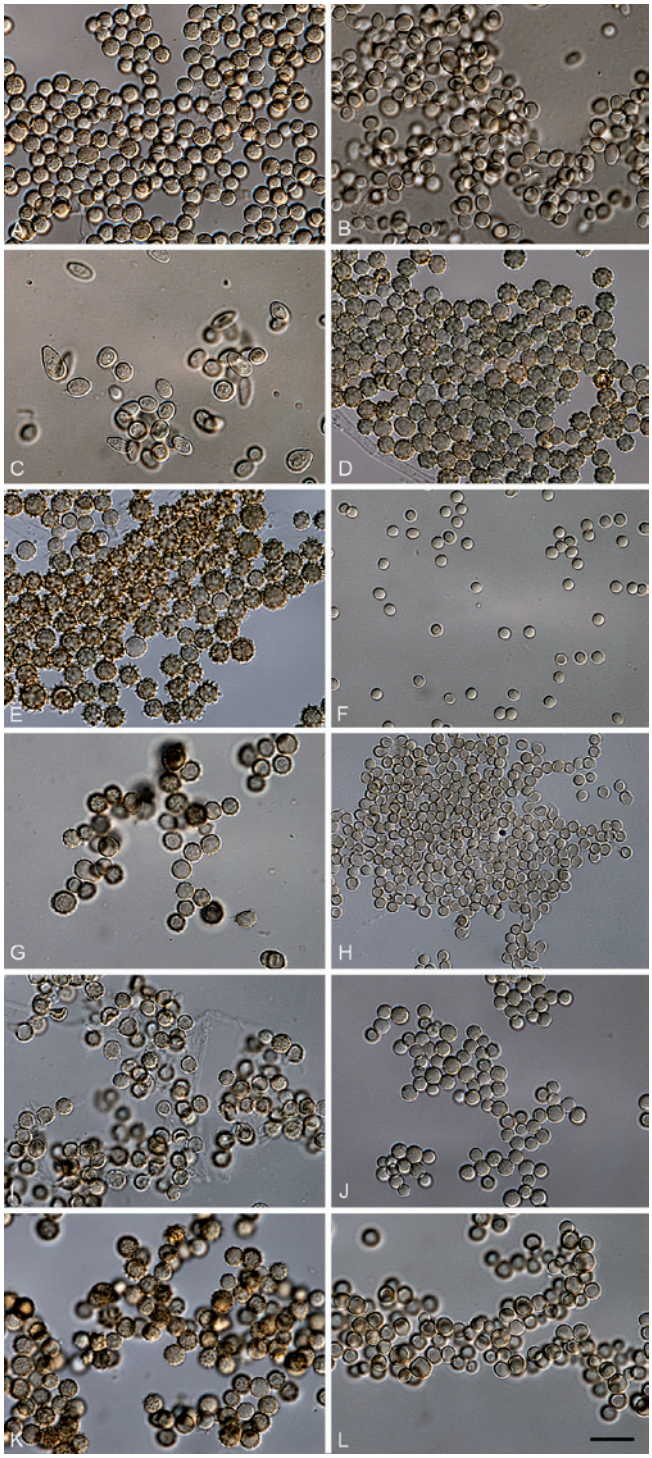

Fig. 13. Conidia of the various species of section *Flavi*. A. *Aspergillus flavus* 100927, B. *A. avenaceus* 109.46, C. *A. coremiiformis* 553.77, D. *A. arachidicola* 117610, E. *A. caelatus* 763.27, F. *A. lanosus* 650.74, G. *A. bombycis* 117187, H. *A. leporis* 151.66, I. *A. nomius* 119388, J. *A. minisclerotium* 117635, K. *A. pseudocaelatus* 117616, L. *A. parvisclerotigenus* 121.62.

in the food industry for the production of soy sauce (known as red Awamori koji) (Jong & Birmingham 1992) and in the fermentation industry for the production of various enzymes, including amylases, proteases, and xylanolytic enzymes (Ferreira *et al.* 1999, Moreira *et al.* 2004). Recently, *A. tamarii* has also been identified as a cause of human keratitis in Southern India (Kredics *et al.* 2007), and *A. tamarii* spores were suggested as important sources of allergens present in the air (Vermani *et al.* 2010). Although *A. caelatus* was found to be very similar to *A. tamarii* morphologically, *A. caelatus* isolates were found not to produce cyclopiazonic acid, in contrast with *A. tamarii* isolates (Horn 1997, Ito *et al.* 1999). *Aspergillus*

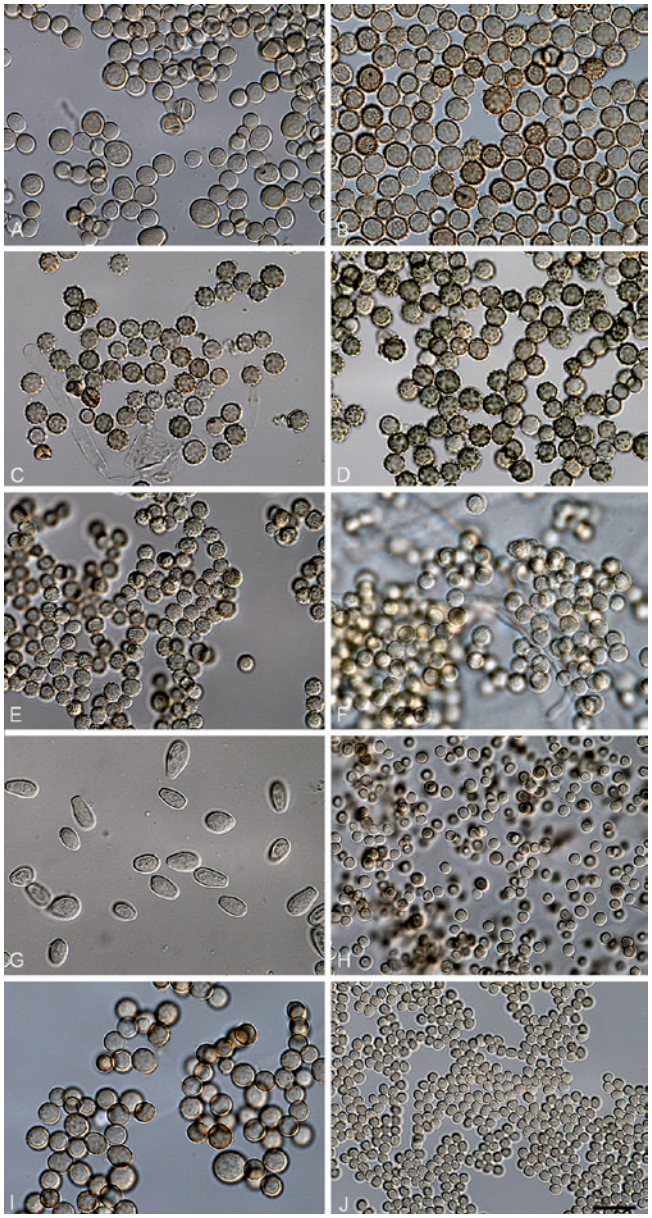

Fig. 14. Conidia of the various species of *section Flavi*. A. *Aspergillus oryzae* 100925, B. *A. pseudotamarii* 766.97, C. *A. sojae* 100928, D. *A. parasiticus* 100926, E. *A. nomius* 260.88, F. *A. pseudonomius* 119388, G. *A. togoensis* 272.89, H. *Petromyces alliaceus* 110.26, I. *A. tamarii* 104.13, J. *P. albertensis* ATCC 58745.

terricola and its subspecies were originally placed into section *Wentii* by Raper & Fennell (1965). Later *A. terricola* together with *A. flavofurcatis* and *A. tamarii* were placed into an "*A. tamarii* species group" by Kozakiewicz (1989). Sequence data indicate that these isolates belong to the same species. *Aspergillus pseudotamarii* (Ito *et al.* 2001) is an effective producer of B-type aflatoxins but the importance for mycotoxin occurrence in foods is unknown. The closely related species *A. tamarii* is not able to produce aflatoxins, despite several reports claiming this (Goto *et al.* 1996, Klich *et al.* 2000). *Aspergillus pseudocaelatus* is represented by a single isolate that came from a *Arachis burkartii* leaf from Argentina. This species produces both G- and B-type aflatoxins, and cyclopiazonic acid.

The "*A. flavus*" clade includes species characterised with Q-10(H_2) as their main ubiquinone, and conidial colours in shades of green, and several isolates produce dark sclerotia. *Aspergillus flavus* is the most common species producing aflatoxins (Sargeant *et al.* 1961), occurring in most kinds of foods in tropical countries. This species is very common on maize, peanuts and cottonseed,

Fig. 15. *Aspergillus togoensis* (CBS 272.89). A–B. Synnemata. C–E. Conidiophores, F. Conidia. Scale bars = 10 µm.

and produces only B-type aflatoxins. It has been estimated that only about 30–40 % of known isolates produce aflatoxin. Because of its small spores and its ability to grow at 37 °C, it can also be pathogenic to animals and humans. Infection by *A. flavus* has become the second leading cause of various forms of human aspergillosis (Hedayati *et al.* 2007, Pasqualotto & Denning 2008, Krishnan *et al.* 2009). *Aspergillus flavus* populations are genetically and phenotypically diverse (Geiser *et al.* 2000) with some isolates producing conidia abundantly, produce large (L) sclerotia, and variable amounts of aflatoxins, while another type produces abundant, small (S) sclerotia, fewer conidia and high levels of aflatoxins (Cotty 1989). The S-type isolates predominated in both soil and maize samples within aflatoxicosis outbreak regions, while the L strain was dominant in non-outbreak regions of Kenya (Probst *et al.* 2010). A related type, *A. oryzae* is atoxigenic and has been used as a source of industrial enzymes and as a koji (starter) mold for Asian fermented foods, such as sake, miso, and soy sauce (van den Broek *et al.* 2001). Although several lines of evidence suggest that *A. oryzae* and *A. sojae* are morphological variants of *A. flavus* and *A. parasiticus*, respectively, it was suggested that these taxa should be retained as separate species because of the regulatory confusion that conspecificity might generate in the food industry (Geiser *et al.* 1998b). *Aspergillus oryzae* isolates carry various mutations in the aflatoxin biosynthetic gene cluster resulting in their inability to produce aflatoxins (Tominaga *et al.* 2006). Particularly, the *aflR* gene is absent or significantly different in some *A. oryzae* strains compared to *A. flavus* (Lee *et al.* 2006). *Aspergillus oryzae* strains can be classified into three groups according to the structure of the aflatoxin biosynthesis gene cluster (Tominaga *et al.* 2006). Group 1 includes strains which has all aflatoxin biosynthesis gene orthologs, group 2 has the region beyond the *ver1* gene deleted, and group 3 has the partial aflatoxin gene cluster up to the *vbs* gene (Chang *et al.* 2009). Isolates assigned to groups 2 and 3 obviously cannot produce aflatoxins due to the loss of part of the gene cluster. Regarding group 1 isolates, the expression level of the *aflR* gene is extremely low, and no expression of several biosynthetic genes (*avnA, verB, omtA, vbs*) was observed. Recent studies clarified that amino-acid substitutions in *AflJ* gene induce inactivation at the protein level (Kiyota *et al.* 2011). Genome sequences of both *A. oryzae* and *A. flavus* are available (Machida *et al*. 2005, Chang & Ehrlich 2010, http://www.aspergillusflavus.org/genomics/).

The genomes of both species are about 37 Mb and consist of 8 chromosomes. A comparative analysis of *A. oryzae* and *A. flavus* genomes revealed striking similarities between them. An

Table 2. Extrolite profiles of species assigned to *Aspergillus* section *Flavi*.

Species	Occurrence	Extrolites produced	Reference
A. arachidicola	Argentina	Aflatoxins B_1,B_2 & G_1,G_2	Pildain *et al.* (2008)
		Aspergillic acid	Pildain *et al.* (2008)
		Chrysogine	Pildain *et al.* (2008)
		Ditryptophenaline	This study
		Kojic acid	Pildain *et al.* (2008)
		Parasiticolides	Pildain *et al.* (2008)
A. avenaceus	UK, USA	Avenaciolide	Brookes *et al.* (1963)
		Aspirochlorine	This study
A. bombycis	Indonesia, Japan	Aflatoxins B_1,B_2 & G_1,G_2	Peterson *et al.* (2001)
		Aspergillic acid	This study
		Kojic acid	This study
A. caelatus	Japan, USA	Aspirochlorin	Pildain *et al.* (2008)
		Kojic acid	Frisvad & Samson (2000)
		Tenuazonic acid	This study
A. coremiiformis	Ivory Coast	Indol alkaloids (not structure elucidated)	This study
A. flavus	Worldwide	Aflatoxins B_1 & B_2	Varga *et al.* (2009)
		Aflatrem	Gallagher & Wilson (1978)
		Aflavarins	TePaske *et al.* (1992)
		Aflavazol	TePaske *et al.* (1990)
		Aspergillic acid	White & Hill (1943)
		Aspergillomarasmines A & B	Haenni *et al.* (1965)
		Cyclopiazonic acid	Luk *et al.* (1977)
		Ditryptophenaline	Springer *et al.* (1977)
		Kojic acid	Birkinshaw *et al.* (1931)
		Miyakamides*	Shiomi *et al.* (2002)
		3-Nitropropionic acid	Bush *et al.* (1951)
		Paspalinine	Cole *et al.* (1981)
A. lanosus	India	Ochratoxins A & B*	Baker *et al.* (2003)
			Palumbo *et al.* (2007)
		Griseofulvin	Frisvad & Samson (2000)
		Kojic acid	Frisvad & Samson (2000)
A. leporis	USA	Antibiotic Y	Frisvad & Samson (2000)
		Kojic acid,	Frisvad & Samson (2000)
		Leporin A	TePaske *et al.* (1991)
		Pseurotin	Frisvad & Samson (2000)
A. minisclerotigenes	Argentina, Australia, Nigeria, USA	Aflatoxins B_1,B_2 & G_1,G_2	Pildain *et al.* (2008)
		Aflavarins	Pildain *et al.* (2008)
		Aflatrems	Pildain *et al.* (2008)
		Aflavinins	Pildain *et al.* (2008)
		Aspergillic acid	Pildain *et al.* (2008)
		Cyclopiazonic acid	Pildain *et al.* (2008)
		Paspalinine	Pildain *et al.* (2008)
A. nomius	Brazil, India, Japan, Thailand, USA	Aflatoxins B_1,B_2 & G_1,G_2	Kurtzmann *et al.* (1987)
		Aspergillic acid	Frisvad & Samson (2000)
		Aspernomine	Staub *et al.* (1992)
		Kojic acid	Frisvad & Samson (2000)
		Nominine	Gloer *et al.* (1989)

Table 2. (Continued).

Species	Occurrence	Extrolites produced	Reference
A. nomius		Paspaline	Staub *et al.* (1993)
		Pseurotin	Frisvad & Samson (2000)
		Tenuazonic acid	Frisvad & Samson (2000)
A. oryzae	China, Japan	Asperfuran	Pfefferle *et al.* (1990)
		Asperopterin A & B*	Matsuura *et al.* (1972)
		Aspirochlorin	Sakata *et al.* (1983)
		Cyclopiazonic acid	Orth (1977)
		Kojic acid	Birkinshaw *et al.* (1931)
		Kojistatin*	Sato *et al.* (1996)
		3-nitropropionic acid	Nakamura & Shimoda (1954)
			Tamogami *et al.* (1996)
		Sporogen AO-1*	Nonoka *et al.* (1997)
		TMC-2A, B, C*	Asai *et al.* (1998)
A. parasiticus	Australia, India, Japan, South America, Uganda USA	Aflatoxins B_1,B_2 & G_1,G_2	Schroeder (1966)
		Aspergillic acid	Assante *et al.* (1981)
		Aspersitin*	Hamasaki *et al.* (1975)
		Kojic acid	Birkinshaw *et al.* (1931)
		Parasperone and ustilaginoidin C*	Brown *et al.* (1993)
		Parasitenone*	Son *et al.* (2002)
		Parasiticolide	Büchi *et al.* (1983)
		Sequoiatones*	Stierle *et al.* (1999, 2001)
		Sequoiamonascins*	Stierle *et al.* (2003)
A. parvisclerotigenus	Nigeria	Aflatoxins B_1,B_2 & G_1,G_2	Frisvad *et al.* (2005)
		Aflatrem	Frisvad *et al.* (2005)
		Aflavarin	Frisvad *et al.* (2005)
		Aspirochlorin	Frisvad *et al.* (2005)
		Cyclopiazonic acid	Frisvad *et al.* (2005)
		Kojic acid	Frisvad *et al.* (2005)
		Paspaline	Frisvad *et al.* (2005)
A. pseudocaelatus	Argentina	Aflatoxins B_1,B_2 & G_1,G_2	This study
		Cyclopiazonic acid	This study
		Kojic acid	This study
A. pseudonomius	USA	Aflatoxin B_1	This study
		Chrysogine	This study
		Kojic acid	This study
A. pseudotamarii	Argentina, Japan	Aflatoxin B_1,B_2	Ito *et al.* (2001)
		Cyclopiazonic acid	Ito *et al.* (2001)
		Kojic acid	This study
A. sojae	China, India, Japan	Asperfuran	This study
		Aspergillic acid	Pildain *et al.* (2008)
		Aspirochlorin	This study
		Chrysogine	This study
		Kojic acid	Tanaka *et al.* (2002)
A. tamarii	Worldwide (mostly warmer climates)	Aspirochlorin	Berg *et al.* (1976)
		(-)-canadensolide*	Berg *et al.* (1976)
		Cyclopiazonic acid	Dorner (1983)
		Fumigaclavine A*	Jahardhanan *et al.* (1984)

Table 2. (Continued).

Species	Occurrence	Extrolites produced	Reference
A. tamarii		Kojic acid	Birkinshaw *et al.* (1931)
		Speradine A	Tsuda *et al.* (2003)
A. togoensis	Central Africa	Aflatoxin B_1	Rank *et al.* (2011)
		Sterigmatocystin	Wicklow *et al.* (1989)
A. alliaceus	Worldwide (Argentina, Australia, Canada, Egypt, France, Greece, Hungary, Lybia, Mexico, Netherlands, New Zealand, Russia, Saudi Arabia, Spain, Tunisia, Turkey, UK, USA)	Asperlicins	Liesch *et al.* (1985)
		Isokotanins	Laakso *et al.* (1994)
		Nominine	Laakso *et al.* (1994)
		Ochratoxin A & B	Ciegler (1972)
		Paspaline	Laakso *et al.* (1994)

*We did not detect these compounds in any strains examined in this study.

array based genome comparison found only 43 genes unique to *A. flavus* and 129 genes unique to *A. oryzae* (Georgianna & Payne 2009). *A.oryzae sensu stricto* has been isolated from koji fermentations used for miso, sake and other Japanese, Korean and Japanese fermented products. Sometimes the species has been reported from cereals, soil etc., and it is possible that all these isolates are just floccose variants of *A. flavus*. Therefore the report of aspergillomarasmin, miyakamides, asperopterins etc. from *A. oryzae*, may actually be from *Aspergillus flavus* (see Table 2). The genome sequenced strain of *A. oryzae* (RIB 40) (Machida *et al.* 2005) was isolated from cereals and probably not from industrial settings, so it is possible that this isolate is a brownish to yellowish green spored variant of *A. flavus* too. Figure 16 illustrates the morphology of the ex-type strain of *A. oryzae* (CBS 100925) showing the typical feature of a floccose strain with less abundant sporulation. Conidiophores produce aberrant conidiogenous structures with elongated or inflated phialides and metulae. Conidia are smooth-walled and subglobose to ellipsoidal. Figure 17 shows the strain of *A. oryzae* (RIB 40) with yellow green colonies and a rich sporulation. This strain also produces abundantly sclerotia which are absent in CBS 100925. Conidiophores of RIB 40 are typical bisteriate with regular shaped conidiogenous structures producing globose, smooth to finely roughened conidia. Phenotypically these two strains are distinct and it would be recommendable to genome sequence an *Aspergillus oryzae* strain used for koji fermentation also, for example the ex-type culture.

Regarding the evolutionary origins of *A. oryzae* and *A. flavus*, Chang *et al.* (2009) suggested that, based on the genetic diversity in the region neighbouring the cyclopiazonic acid biosynthesis gene cluster, *A. oryzae* most likely descended from an ancestor that was the ancestor of *A. minisclerotigenes* or *A. parvisclerotigenus* producing both B- and G-type aflatoxins, while *A. flavus* descended from an ancestor of *A. parasiticus*.

Population genetic analyses of restriction site polymorphisms and DNA sequences of several genes indicated that *A. flavus* isolates fell into two reproductively isolated clades (groups I and II). A lack of concordance between gene genealogies among isolates in group I suggested that *A. flavus* has a recombining population structure (Geiser *et al*. 1998, 2000). Regarding the distribution of the mating type genes in *A. flavus* populations, there was no significant difference in the frequency of the two mating types for *A. flavus* (and *A. parasiticus*) in either vegetative compatibility groups (VCG) or haplotype clone-corrected samples. The existence of both mating type genes in equal proportions in these populations together with the observed expression of these genes indicated the possible existence of a sexual state in *A. flavus* (Ramirez-Prado *et al.* 2008). The presence of mating type genes have also been observed in *A. oryzae* isolates (Chang & Ehrlich 2010). Recently the sexual stage of *A. flavus* has been described under the name of *Petromyces flavus* (Horn *et al*. 2009a, 2009b). However, in another study the distribution of mating type genes was uneven within an *A. flavus* population collected from maize fields in Southern Hungary, indicating that the given population reproduces primarily clonally (Tóth B. *et al.* in preparation). Indeed, population genetic analyses of molecular data confirmed that this population is a clonal one (data not shown). Sweany (2010) also observed uneven distribution of mating type genes in *A. flavus* isolates collected from maize with MAT1-2 being dominant (96 %), while the distribution of mating type genes was more balanced in soil isolates (48 % with MAT1-1, and 52 % with MAT1-2 idiomorphs). She also observed that the isolates belonging to different vegetative incompatibility groups of *A. flavus* almost exclusively carried either one or the other mating type gene (Sweany 2010). Differences between the corn and soil populations were suggested to indicate that not all soil isolates are as capable of infecting corn, and that some isolates have become specialised to infect corn.

Multilocus sequence data indicated that several species assigned to section *Flavi* are synonyms of *A. flavus*, including *A. flavus* var. *columnaris, A. kambarensis*, *A. fasciculatus*, *A. thomii* and *A. subolivaceus* (Figs 1–3). Although Peterson (2008) observed that *A. subolivaceus* formed a separate lineage distinct from *A. flavus* based on sequence data of two loci, it could not be distinguished by any other means from *A. flavus* isolates. Some of these species have also been found to be synonyms of *A. flavus* based on sequence analysis of part of their 18 S and 26 S rRNA genes (Nikkuni *et al.* 1998, Peterson 2000). Strains of *A. flavus* var. *columnaris* produce pronounced conidial columns, and most strains accumulate aflatoxin B_2 only. It appears that certain mutations have induced this characteristic phenotype. The *A. kambarensis, A. fasciculatus, A. thomii* and *A. subolivaceus* ex-type strains could not produce aflatoxins, showing that aflatoxin ability can easily be lost in soil strains of *A. flavus*.

Many reports indicate that certain *A. flavus* strains, including micro-sclerotial strains, and strains listed as intermediate between *A. flavus* and *A. parasiticus* can also produce type G aflatoxins (Codner *et al.* 1963, Hesseltine *et al.* 1970, Cotty & Cardwell 1999, Begum & Samajpati 2000). One group of these isolates have been named previously as *A. flavus* var. *parvisclerotigenus* (Saito *et al.* 1986, Saito & Tsuruta 1993), and later raised to species status as *A. parvisclerotigenus* (Frisvad *et al.* 2005). The type strain of *A. parvisclerotigenus* (CBS 121.62 = NRRL A-11612 = IBT 3651 = IBT 3851) was isolated from peanut in Nigeria, and this species has

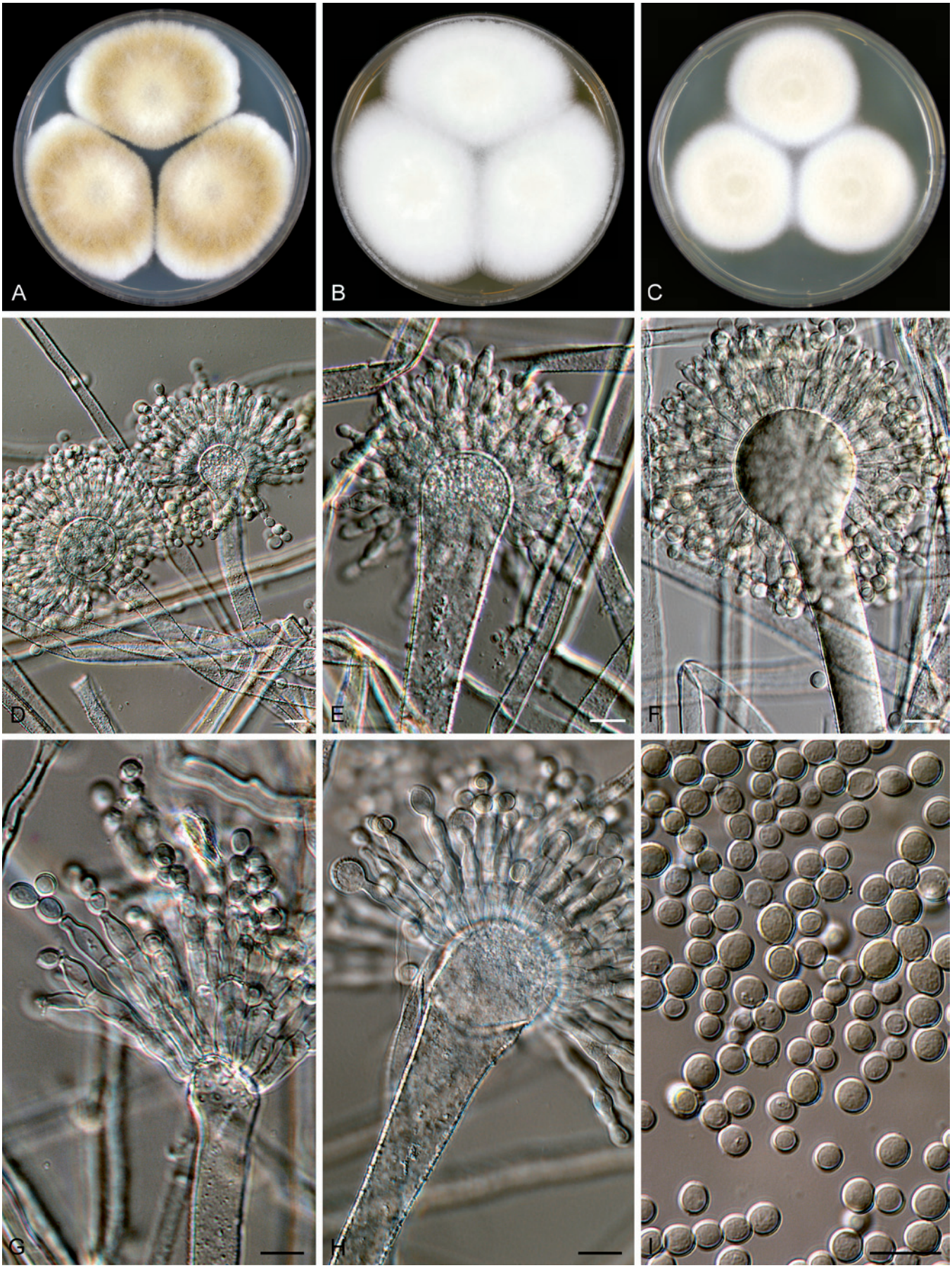

Fig. 16. *Aspergillus oryzae* (ex-type CBS 100925). A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. YES, D–I. Conidiophores and conidia. Scale bars = 10 µm.

also been identified in grain samples came from Nigeria and Ghana (Perrone *et al.* 2009).

Another group of *A. flavus*-related isolates producing both B- and G-type aflatoxins has also been described as *A. minisclerotigenes*. This species was originally isolated from Argentinean peanuts and had small sclerotia and produced aflatoxins B_1, B_2, G_1, G_2, aspergillic acid, cyclopiazonic acid, kojic acid, parasiticolides and several other extrolites (Pildain *et al.* 2008, Table 2). One of the strains

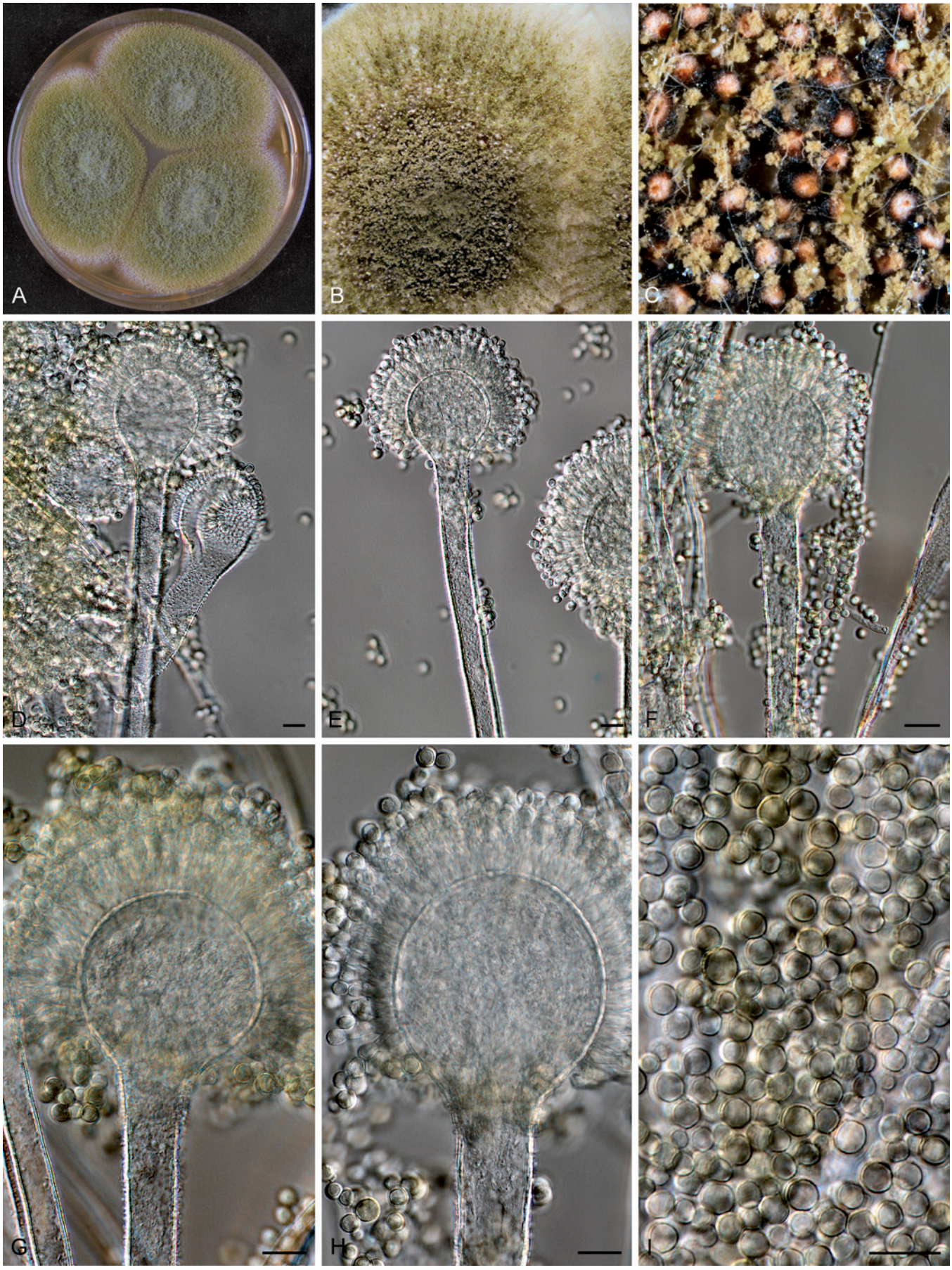

Fig. 17. *Aspergillus oryzae* (RIB 40). A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. Sclerotia, D–I. Conidiophores and conidia. Scale bars = 10 µm.

listed by Hesseltine *et al.* (1970), NRRL A-11611 = NRRL 6444 also produced aflatoxins B_1, B_2, G_1 and G_2, aflatrem, aflavinines, aspergillic acid, cyclopiazonic acid, parasiticolides, kojic acid, aspergillic acid, paspaline, paspalinine and emindole SB and is an *A. minisclerotigenes*. *Aspergillus parvisclerotigenus* has an extrolite profile very similar to that of *A. minisclerotigenes*, but in contrast

with the Argentinean strains, it also produces parasiticolides, and compound A 30461 (aspirochlorin = oryzachlorin; Table 2). Based on the molecular studies, *A. minisclerotigenes* seems to be quite widespread occurring in Argentina, USA, Nigeria and Australia as well (Pildain *et al.* 2008). Recently, Damann *et al.* (2010) observed sexual recombination between compatible partners of Australian isolates assigned to *A. flavus* groups I and II by Geiser *et al.* (1998). Further studies are needed to clarify the significance of these findings.

A third group of microsclerotial strains, represented by NRRL 3251, actually produces only B-type aflatoxins, but are, except being the S-type, typical *A. flavus*. Even though most strains of *A. flavus* produce large sclerotia, a smaller number of strains can produce small sclerotia. Thus at least three taxa can produce small sclerotia.

Many other isolates producing both aflatoxins B and G and bearing small sclerotia have been reported to date (Bayman & Cotty 1993, Saito & Tsurota 1993, Egel *et al.* 1994, Cotty & Cardwell 1999, Frisvad *et al.* 2005). Isolates came from maize, almond and cocoa beans and assigned to *A. flavus* based on either morphological or ITS sequence data have also been found to belong to different chemotypes based on their abilities to produce aflatoxins B_1, B_2, aflatoxin G_1, G_2 and cyclopiazonic acid (Razzaghi-Abyaneh *et al.* 2006, Giorni *et al.* 2007, Sanchez-Hervas *et al.* 2008, Rodrigues *et al.* 2009). Recently, Donner *et al.* (2009) found that about 8 % of the *Aspergillus* section *Flavi* isolates collected in maize fields in Nigeria produce small sclerotia and both B- and G-type aflatoxins. These isolates which presumably belong to *A. minisclerotigenes* together with *A. parasiticus* were suggested to be the greatest contributors to aflatoxin contamination of maize in regions where they occurred (Donner *et al.* 2009). Further studies are necessary to assign these isolates to species.

Another important aflatoxin producer, *Aspergillus parasiticus* occurs rather commonly in peanuts, and almonds (Rodrigues *et al.* 2009), but is apparently quite rare in other foods (*e.g.* on dried figs; Oktay *et al.* 2009). It is more restricted geographically as compared to *A. flavus*. *Aspergillus parasiticus* produces both B- and G-type aflatoxins (Sargeant *et al.* 1963), and virtually all known isolates are toxigenic. Linkage disequilibrium analyses of variation across 21 intergenic regions also revealed several distinct recombination blocks in *A. parasiticus*, and recombination events have also been observed between different vegetative compatibility groups (Carbone *et al.* 2007). The even distribution of the mating type genes in *A. parasiticus* populations was also indicative of the presence of a cryptic sexual stage (Ramirez-Prado *et al.* 2008). Recently, crosses between strains carrying opposite mating-type genes resulted in the development of ascospore-bearing ascocarps embedded within stromata. Sexually compatible strains belonged to different vegetative compatibility groups (Horn *et al.* 2009b). The sexual state of *A. parasiticus* has been described as *Petromyces parasiticus* (Horn *et al*. 2009c).

Nontoxigenic *A. flavus* and *A. parasiticus* isolates are used to control aflatoxin levels in various agricultural products. Great success in reducing aflatoxin contamination have been achieved by application of nontoxigenic strains of *A. flavus* and *A. parasiticus* in fields of cotton, peanut, maize and pistachio (Brown *et al*. 1991, Pitt & Hocking 2006, Dorner 2008). Significant reductions in aflatoxin contamination in the range of 70 %–90 % have been observed consistently by the use of nontoxigenic *A. flavus* and *A. parasiticus* strains (Pitt & Hocking 2006, Dorner 2008, Yin *et al.* 2008). Actually, two products of nontoxigenic strains have received U.S. Environmental Protection Agency (EPA) registration as biopesticides to control aflatoxin contamination in cotton and peanuts in several states of USA (Dorner 2008). This strategy is based on the application of nontoxigenic strains to competitively exclude naturally toxigenic strains in the same niche and compete for crop substrates. However, the discovery of a sexual cycle in *A. flavus* and in *A. parasiticus* raised concerns about the safety of these products. Indeed, Olarte *et al.* (2010) found that a single generation of sexual reproduction between a nonaflatoxigenic *A. flavus* isolate containing a single mutation in the aflatoxin biosynthesis gene cluster and an aflatoxigenic parent can restore aflatoxin production due to a crossing over within the aflatoxin biosynthesis gene cluster. In other crosses involving strains with either a partial aflatoxin gene cluster or strains missing the entire cluster and an aflatoxigenic *A. flavus* strain also regained toxicity via independent assortment of chromosomes, questioning the safety of using non-aflatoxigenic *A. flavus* or *A. parasiticus* strains for lowering aflatoxin levels in agricultural products. *Aspergillus toxicarius*, which also produces B- and G-type aflatoxins (Murakami *et al.* 1966, Murakami 1971), was suggested to be conspecific with *A. parasiticus* by Kozakiewicz (1989), which view is supported by the sequence data. *Aspergillus terricola* var. *americanus* (which does not produce aflatoxins!) and *A. parasiticus* var. *globosus* (which produces all the known aflatoxins) could also not be distinguished from *A. parasiticus* by neither phylogenetic analysis of multilocus sequence data nor by extrolite profiles indicating that these are also synonyms of *A. parasiticus* (Figs 1–3). *Aspergillus sojae* is the domesticated variety of *A. parasiticus*, which can hardly be distinguished from it apart from its inability to produce aflatoxins (Rigó *et al.* 2002, Chang *et al.* 2007). The lack of aflatoxin-producing ability of some *A. sojae* isolates results primarily from an early termination point mutation in the pathway-specific *AflR* regulatory gene, which causes the truncation of the transcriptional activation domain of *AflR* and the abolishment of interaction between *AflR* and the *AflJ* co-activator. In addition, a defect in the polyketide synthase gene also contributes to its nonaflatoxigenicity (Chang *et al.* 2007). Recently, Garber *et al.* (2010) identified *A. parasiticus* lineages associated with maize and peanut cultivation in USA, Asia and Africa, and a presumably new species with an ancient, global and almost exclusive association with sugarcane (*Saccharum* sp.). Again a soil-borne form of *A. parasiticus, A. terricola* var. *americanus*, and the domesticated forms (*A. sojae*) cannot produce aflatoxins similar to the examples in *A. flavus*.

Aspergillus arachidicola was isolated from leaves of *Arachis glabrata* in Argentina, and produce aflatoxins B_1, B_2, G_1 and G_2, aspergillic acid, chrysogine, aspirochlorin, parasiticolide, ditryptophenaline and the extrolite NO2. All strains had a floccose colony texture, a conidium colour similar to *A. flavus* but, except for the production of chrysogine by most isolates, they exhibited extrolite profiles similar to those of *A. parasiticus* isolates (Pildain *et al.* 2008, Table 2).

Aflatoxins have been shown to be produced by *A. flavus*, *A. parasiticus* (Codner *et al.* 1963, Schroeder 1966), *A. nomius* (Kurtzman *et al.* 1987), *A. pseudotamarii* (Ito *et al.* 2001), *A. bombycis* (Peterson *et al.* 2001), *A. toxicarius* (Murakami 1971, Murakami *et al.* 1982, Frisvad *et al.* 2005), *A. parvisclerotigenus* (Saito & Tsurota 1993, Frisvad *et al.* 2005), *A. minisclerotigenes, A. arachidicola* (Pildain *et al.* 2007) and *A. pseudonomius* and *A. pseudocaelatus* in *Aspergillus* section *Flavi.* Aflatoxin-producing species are scattered throughout the phylogenetic trees indicating that aflatoxin-producing ability was lost (or gained) several times during evolution.

ACKNOWLEDGEMENTS

We thank Dr Uwe Braun for the Latin diagnosis and advice on nomenclatural issues. Dr Belen Pildain kindly provided the strain of *A. pseudocaelatus*. Mr Dae-ho Kim (Korean Agricultural Culture Collection), Suwon, South Korea, kindly helped us with photographing the colonies and conidia of the species in section *Flavi*.

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